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TEST METHODS FOR ASSESSING THE EFFECTS OF CHEMICALS ON PLANTS

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TEST METHODS FOR ASSESSING THE EFFECTS OF CHEMICALS ON PLANTS

JUNE 1975

FINAL REPORT

OFFICE OF TOXIC SUBSTANCES
ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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16. ABSTRACT

This report is the result of a survey undertaken to develop a series of acceptable test protocols for assessing the effects of chemicals on plants. Plant exposure to air, soil, and water contaminants were considered. Test species were selected on the basis of physiology, anatomy, importance as crops, and their susceptibility to chemicals. Bioassay methods were chosen because they are most representative of natural conditions. The recommended sequence of test procedures includes: growth chamber or laboratory testing, greenhouse testing, field plot testing, progeny testing and, finally, population studies in the ecosystem. It is suggested that the extent of testing should depend upon the quantity of the chemical to be manufactured and its potential hazard to the environment.

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This report has been reviewed by The Office of Toxic Substances, EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

FOREWORD

The work described in this report was performed by the Environmental Protection Group, Science Information Services Department, The Franklin Institute Research Laboratories, under U.S. Environmental Protection Agency Contract # No. 68-01-2249. The study was conducted under the direction of Messrs. Alec Peters, Department Director, and Bernard E. Epstein, Manager of the Environmental Protection Group; Mr. Richard Rubinstein was the project manager. Dr. Elton R. Homan of the Office of Toxic Substances, U.S. Environmental Protection Agency, was the Project Officer.

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I. INTRODUCTION

The purpose of this report is to indicate suitable test species and methods for the determination of toxicity on plants. Plant exposure to air, soil, and water contaminants have been considered. The loss of a volatile compound into the atmosphere in the form of a gas or aerosol during manufacture may indicate a potential hazard to plants via the vapor phase, thereby necessitating air pollution testing.

Material was obtained both from published literature and unpublished sources. Many knowledgeable members of the scientific community were interviewed.

The implementation of the proposed Toxic Substances Control Act will require safety evaluation of most substances commercially manufactured by the United States chemical industry. In the utilization of some substances, every pound of material can be accounted, retained or converted with no environmental release. Other substances will be used under circumstances where significant quantities will be released into the environment. Inevitably, some losses into the environment will be sustained during manufacture. The modes of loss are described in Table 1.1.

Table 1.1 Soil, Water, and Atmospheric Pollutant Effects on Terrestrial Plant Growth

I. Sources of toxicants

    A. Point source release.

        1. Air transport

        - a. Direct effects on plants
        - b. Indirect effects by
            - (1) Uptake from soil
            - (2) Uptake from water
    - 2. Water transport
    - 3. Direct application
        - a. liquid, i.e., spray irrigation.
        - b. solids or semi-solids.
- B. Non-point sources.
    - 1. Loss from manufacture sites.
    - 2. Release following consumption and utilization.

The ease of detection of phytotoxic effects with vascular plants depends on severity and morphological expression. Gross morphologic effects are usually readily apparent. Subtle changes in physiology affecting crop yield can be detected only through ecological study of the various populations within the community. This is also applicable to changes in competition and reproductive succession in native plant communities.

The extent of required testing should depend on the amount of material manufactured and released into the environment. A material that is manufactured in small quantities with complete conversions or recovery and no environmental release may require no plant testing. With an increase in the quantity manufactured and projected environmental release, the degree of plant toxicity testing should also increase.

Ideally, a series of test protocols should be developed and listed as acceptable evaluation procedures. Because of the great variability within the plant world, these should represent guidelines and acceptable methods, not as rigid either/or procedures. The procedure and techniques should begin in a rather uncomplicated fashion and increase in complexity and sophistication with testing requirements.

Some herbicide chemists and plant physiologists believe that no procedure is a precise guide to the potential for phytotoxicity. Technically, this is correct. Plant species, age, dosage, chemical formulation, exposure site and other factors will influence plant response. These considerations might be taken to indicate that any efforts to develop test protocols would be futile. However, even an inadequate effort would be better than none, and a preliminary evaluation could provide a basis for further work. It is essential to recognize that simple laboratory or greenhouse procedures in phytotoxicity evaluation represent a commencement in investigation. The end point in testing is dependent on the extent and manner of chemical release.

The majority of plant toxicity studies has been performed by herbicide chemists, physiologists, and air pollution specialists. The techniques described and species are therefore those used for studying known pollutants and chemicals synthesized for their herbicidal (albeit differential) effects.

1.1 TEST METHODS FOR TERRESTRIAL AND AQUATIC POLLUTANTS

The degree of complexity, sophistication, and expense of a particular test should be governed by the chemical's potential hazard to the environment. This includes the amount produced, projected use (industrial or consumer), waste disposal procedures, and the environmental fate of the compound. Therefore, test methods have been arranged in their order of complexity: laboratory (incubator), greenhouse, field plot, and specialized. In the scheme (Figure 1-1, amplified below), the toxicity of the previous test is considered. Therefore, detection of toxicity in an early stage will necessitate intensive testing at complex levels.

The following are sequential procedures for development of terrestrial plant soil and water pollutant hazard evaluation (elementary to complex).

1. Growth chamber or laboratory testing
    a. seedlings in pots.
    b. soil, water, or foliar spray exposure route for test chemical.
    c. standard plant species.
2. Greenhouse testing
    a. seedlings grown to maturity.
    b. soil, water or foliar spray exposure route for test chemical.
    c. standard plant species.
3. Preliminary field plot testing

    - a. seedlings grown to maturity--yield data.
    - b. soil, water or foliar spray exposure route for test chemical.
    - c. standard plant species and selected plant species as likely to occur in release site.

4. Field plot testing
    - a. seedlings grown to maturity--yield data.
    - b. soil, water or foliar spray exposure route for test chemical.
    - c. standard plant species and selected plant species as likely to occur.
    - d. field evaluation on sites of likely release or introduction of test chemical in actual situation.
    - e. multi-year continued testing.
5. Progeny testing-Mutagenicity and F_1 population effects from "selfed" parents where possible.
6. Population studies in natural ecosystems where applicable (especially with non-point source release materials). These would be continuing studies on release sites.

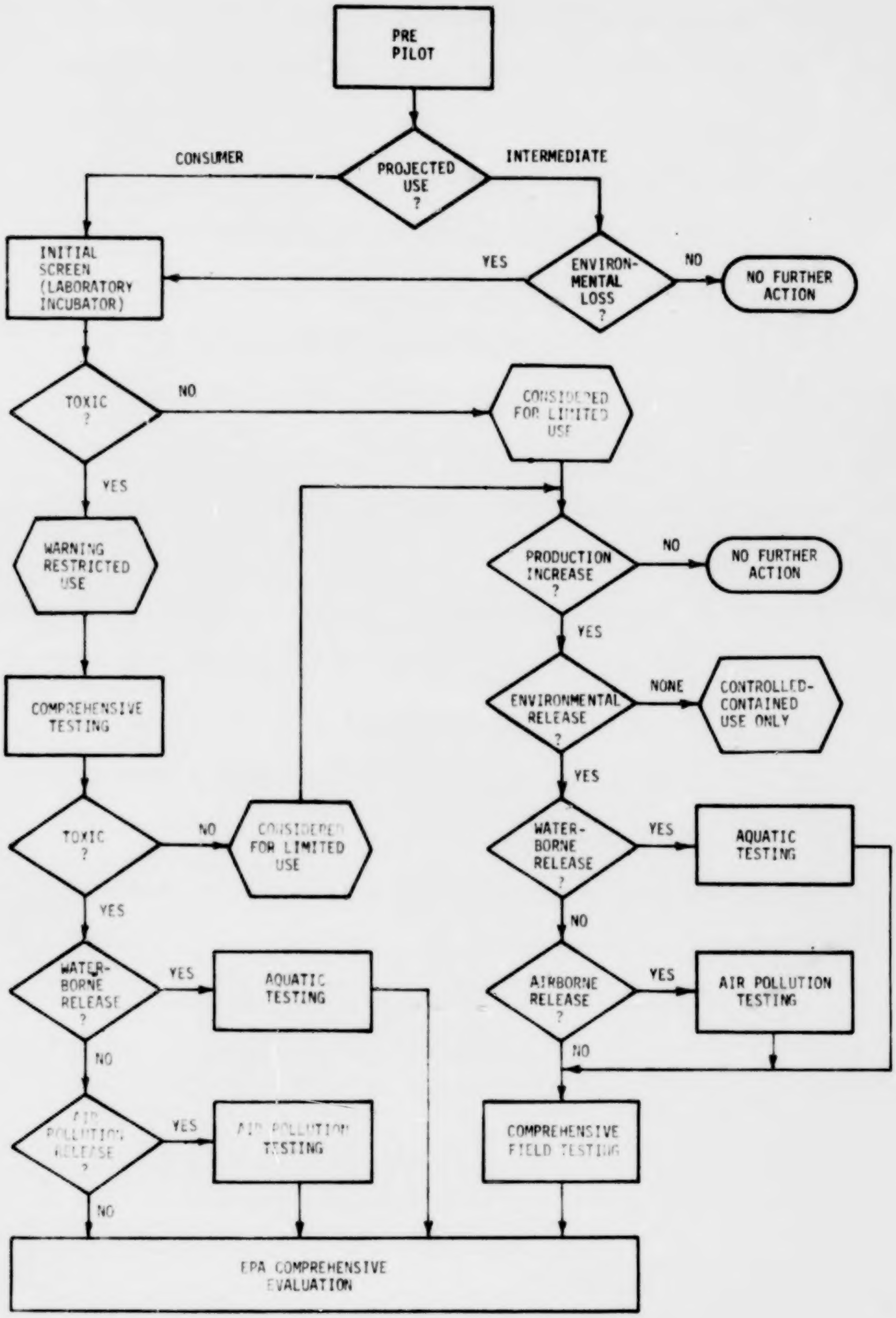
PRE PILOT
PROJECTED USE ?
CONSUMER
INTERMEDIATE
INITIAL SCREEN (LABORATORY INCUBATOR)
YES
ENVIRON-MENTAL LOSS ?
NO
NO FURTHER ACTION
TOXIC ?
NO
CONSIDERED FOR LIMITED USE
YES
WARNING RESTRICTED USE
PRODUCTION INCREASE ?
NO
NO FURTHER ACTION
YES
COMPREHENSIVE TESTING
ENVIRONMENTAL RELEASE ?
NONE
CONTROLLED-CONTAINED USE ONLY
YES
TOXIC ?
NO
CONSIDERED FOR LIMITED USE
WATER-BORNE RELEASE ?
YES
AQUATIC TESTING
YES
NO
WATER-BORNE RELEASE ?
YES
AQUATIC TESTING
AIRBORNE RELEASE ?
YES
AIR POLLUTION TESTING
NO
NO
AIR POLLUTION RELEASE ?
YES
AIR POLLUTION TESTING
COMPREHENSIVE FIELD TESTING
NO
EPA COMPREHENSIVE EVALUATION

Figure 1. Proposed Testing Scheme for Plant Toxicity Testing

1.2 TEST METHODS FOR AIRBORNE POLLUTANTS

Research on the effects of toxic airborne contaminants on plants began about 100 years ago in Tharandt, Germany where damage to forests by smelter fumes came under investigation. A considerable number of studies have been made in subsequent years to identify a number of important atmospheric toxicants of anthropogenic origin. Unfortunately, the toxicants studied are few in number; the most important ones are sulfur dioxide, ozone, fluorides, nitrogen oxides, peroxyacyl nitrates, chlorine, chlorides, ethylene, some particulates, and a few other chemicals, including herbicides. Although these compounds represent the great bulk of those emitted into our atmosphere by industry or by internal combustion engines, they represent only a small percentage of the potentially toxic airborne substances.

Because the number of potentially toxic substances is almost unlimited and there is essentially no body of information available concerning relative toxicities, concentrations, and durations of exposure to produce toxic effects, or a list of the plant receptors that are useful as bio-indicators, only general approaches can be recommended at this time. Each of these is based upon methods that have been employed for the diagnosis or evaluation of injury from the more important air pollutants.

1.2.1 Plants as Biological Monitors of Air Pollution

Both the higher and lower plants have been employed extensively to monitor air pollution near industrial and urban areas. Some plants are extremely susceptible to specific pollutants (e.g., Bel-W_3 tobacco for ozone, lichens for sulfur dioxide, gladiolus for fluoride, etc.). To determine the validity of these and other indicators has taken many years of controlled fumigation studies and field observations. No species of plant can be recommended that would be satisfactory for all potential toxicants and, at our present level of knowledge, a different species of plant cannot be recommended for each potential toxicant. The use of plants as biological monitors would expose the native vegetation in the field to dosages of the potential toxicant that might occur in the atmosphere. This can be done with open-top field chambers, supplied with either the test chemical or unpolluted air. Where the toxic substance is already emitted into the atmosphere, the same chambers may be used to expose native plants to the ambient pollutant and other plants to clean air following selective filtration. Both approaches are presently being used in air pollution research, but have been limited to studies with the more common phytotoxicants.

1.2.2 Effects of Air Pollutants on the Histology of Plants

That air pollutants alter the histology of plant organs has been known from studies at Tharandt, Germany during the latter half of the nineteenth century. Attempts to use comparative histology or histological profiles to diagnose plant injury have been relatively common during the past 20 years. In some cases, ultrastructural changes as seen with the electron microscope have been documented. For the most part, the use of histological profiling to discriminate between the common air pollutants has been only moderately successful, and injuries induced by other factors produce similar histological syndromes.

1.2.3 Effects of Air Pollutants on Metabolism or Metabolite Pools

Although there have been many investigations on alterations in physiological and biochemical processes of plants by air pollutants, no methods of real diagnostic value have been developed even for the common phytotoxic air pollutants. Of the possible methods available, the development of an isozymic profile for a number of phytoxicants might be the most successful. But, even this method would have value only for the few toxicants studies and not for the bulk of potential toxicants.

1.2.4 Effects of Air Pollutants on Cytogenetics

This approach might have value for possible chemical mutagens, but relative utility of various test species has not yet been found. Sparrow, at Brookhaven, however, has developed what may become a very sensitive assay for mutagenesis using the stamen hairs of *Tradescantia*, but this work has not advanced sufficiently to be useful.

1.3 TEST ORGANISM SELECTION

Considerable attention was given to the reasons for selecting any given organism. Respondents in personal interviews differed according to their orientation and organizational mission.

Studies with pesticides and herbicides have generally utilized crop and weed plants. There appears to be a "core" of test crops with some variation according to locale. Generally, industry was more pragmatic in fixing the number of species based on "cash" crops. Industrial protocols were well-defined as to the series of tests to be performed for any given chemical. From academic scientists, there were more variations in both tests and species.

In air pollution testing, the species was determined by the pollutant. These investigators have determined which plants are sensitive to the most common air pollutants and have designed their protocols and tests accordingly.

Aquatic testing was limited mainly to algae and aquatic weeds. Inasmuch as the latter are target organisms, most testing has been done for "efficacy" rather than for plant protection.

Generally, cultivated species selected for study were from uniform strains. Weeds presented a particular problem with respect to uniformity and simulation of natural conditions. Many interviewees responded that weed seeds should be exposed to extreme cold before planting to simulate natural conditions. Cultivated plants however were easily maintained under laboratory conditions. More were selected for economic importance rather than ecological significance. Several were selected for ease of maintenance or testing.

In the description of tests in this report were included those species which have been used and, on occasion, should be used. In general, the most pragmatic considerations have been followed. Plants of economic concern include or represent important crops. However, one must consider the particular community which is threatened. Two important considerations for selection within any mixed plant-animal community are species dominance and position in the animal-plant food chain. Damage to a dominant plant species can result in succesion changes within a community. Destruction of a particular food plant could affect animal as well as plant succession. The uptake, metabolism, and/or biological magnification of a chemical by a plant species also presents a hazard to species in the surrounding communities. Selection of species, strains and stages of development for testing has been based on these considerations. When a large volume pollutant release affects predominant or important species, comprehensive testing of that particular species should be undertaken.

2. SELECTION OF TEST METHODS

2.1 INTRODUCTION

The bulk of the knowledge regarding test procedures for chemical effects on plant growth has arisen as a result of the search for chemical configurations with herbicidal or growth regulatory properties, or a result of investigations on the response of the plants to air pollutants emitted by industries or motor vehicles. Because of the prominence of the screening procedures developed for herbicides, the greater part of this discussion will be directed to the absorption and movement of the substances in plants. Much of this work has been done in the laboratories of the major chemical manufacturing firms throughout the world. Some has been done by public research agencies including the United States Landgrant University Experiment Stations and the U.S.D.A. Agricultural Research Service. Various methods have been published in the scientific periodical literature. Others reside only in the laboratories and libraries of the chemical companies.

An axiom often repeated and demonstrated in this research is: "The correct question must be asked to obtain the correct answer," or stated another way; no single evaluation procedure would have detected the majority of the economically important chemical compounds presently available and registered for use as herbicides and growth regulators in the United States. Some of the most widely used herbicides on the market today were tested and discarded by other firms employing different evaluation screening procedures for physiological activity in plants.

2.2 MECHANISMS OF TOXICANT ACTION

The world-wide searches by pesticide manufacturers for chemical configurations that exhibit physiological activities towards the Tracheophyta, more specifically the Spermatophyta, have elicited hundreds of substances, perhaps thousands, with such effects. Some obviously are herbicidal, others herbistatic, and still others growth regulants in various ways. The overwhelming majority of these configurations are never developed commercially. In the search for this activity most of the firms' research and development divisions immediately evaluate through various screening procedures those chemicals closest at hand. These would include all chemicals manufactured, chemical process intermediates, chemical process by- or waste products, and stock chemicals on the shelves for one reason or another. Through the empirical process many major breakthroughs in herbicide technology have occurred. Once a certain configuration is determined active, then organic specialists rapidly synthesize as many analogues and related compounds as possible seeking the most appropriate specific compound.

In this manner, we believe that in the last 30-year period since the introduction of herbicides into world-wide use, many of the common mechanisms for phytotoxic effects in plants have been elucidated. Thus a review of the herbicide literature dealing with mechanisms of action will shed light on probable mechanisms in the total range of toxic substances. Furthermore, this body of literature points out the fallacy in attempting to use a single screening or evaluation procedure against all kinds of potential toxicants with any hope of success. Hilton et al. (1) points out this multiplicity of mechanisms all of which influence the kinds of testing required to determine effects.

The chemical categories discussed in the following pages illustrate the diversity of principles involved in mechanisms of phytotoxic action. The inclusion or exclusion of a group of toxicants does not necessarily imply greater or lesser significance.

In reference to plants the term growth has been given a variety of meanings, often being used synonymously with yield. Whereas the end product sought by the practical agriculturalist is yield, yield is not growth per se. Instead yield is a result of growth. Growth can be defined better in terms of production of more protoplasm and cellular development, namely: cell division, elongation, and differentiation. These processes bring about an irreversible increase in volume.

Growth rates do not continue at a constant velocity because of hormone inactivation or inhibition. Growth rates may also be affected by the inactivation of phosphate transferring enzyme systems. Shifts in growth from production of vegetative parts to the production of reproductive parts usually results in the reduction or cessation of the former.

Patterns of growth in plants are accompanied by certain gross changes in the proportions of the main chemical constituents of the plant parts. These gross changes, expressed as percentage of dry weight, are primarily shifts in protoplasmic constituents from a high level in rapidly growing tissues to lower levels as these tissues cease growth and their cells differentiate secondary wall materials.

Growth of higher plants from seed to maturity and the production of more seed is the intimate involvement of a number of complex processes. Some of the more readily identifiable processes include imbibition, diffusion, osmosis, active and passive absorption, mineral function, transpiration, chlorophyll and other pigment synthesis, photosynthesis, synthesis of carbohydrates, fats, proteins, vitamins, hormones, etc., digestion, assimilation, translocation, respiration, and reproduction. The processes are interdependent and influenced by environmental, genetic and pathological factors, enzymes, hormones, and vitamins.

Chemicals can affect the growth and development of plants in many diverse ways, ranging from subtle alteration of the normal development of some plants to outright death of others. The means by which chemicals affect the growth and development of plants are diverse, in many instances unknown, and theoretically as numerous as the processes essential to plant life.

It is not necessarily true that all chemicals affect plant growth, or, that those that do affect plant growth do so only in an abnormal or detrimental manner. The agricultural scientist in cooperation with the chemists have learned to produce many chemicals for selective control of certain types of plants in crop production and for the control of other plants for industrial, health, and recreational purposes. Other types of chemicals have been developed that alter plant growth in specific ways, thus are useful for the economic culturing of such species of plants.

The Committee on Plant and Animal Pests of the National Research Council's Agricultural Board considered the question of what effects chemicals that are used for the control of pests may have on host plants (2). Attention was accorded in their study to any secondary effect produced at all stages of plant growth, i.e., seed germination, vegetative development, sexual reproduction, development of storage organs, maturation, harvest and post-harvest behavior, nutritional value, and market quality of fresh and processed food products by such chemicals when used within their normally prescribed dosages for various pest control purposes. Their careful sampling of the vast number of literature references to pesticide trial reports resulted in the substantial listing of over 600 citations that made reference to secondary effects produced by 110 different chemicals used for agricultural purposes. The major secondary effects noted for these chemicals are listed in Table 2.1.

Superficial observations and published literature, stating that there are no effects to the crop plants following application of certain chemical pesticides have led to the widely held assumption that such chemicals do not cause significant effects on plants exposed to them. Thus the information in Table 2.1, in addition to demonstrating the erroneousness of this belief, also emphasizes that not all of the secondary

Table 2.1 Different Types of Secondary Effects Observed in Plants After to a Variety of Agricultural Chemicals

phytotoxicity

decreased and increased phytosynthetic activity

altered chlorophyll content

induced chlorosis due to interaction with minerals or other chemicals

decreased and increased respiration

reduced transpiration

altered carbon dioxide assimilation

enhancement of vegetative growth

stimulated or delayed root development

proliferation and suppression of vegetative growth

affected setting of fruit

affected pollen germination

malformation or atrophy of various plant parts

potentiation of other chemicals

decreased or increased harvestable yield

reduction or increases in size and/or number of fruit

delayed or accelerated fruit maturity

improved appearance of fruit and vegetables

russetting of fruit

discoloration of leaves

absicission of leaves and fruit

hardening and stunting of plants

altered thickness of leaves

altered senescence

resistance and susceptibility to drought

resistance and susceptibility to freezing

susceptibility to scorching or burning of leaves

increases or decreases in plant composition (including carbohydrates, protein, amino acids, lipids, and vitamins)

Table 2.1 Different Types of Secondary Effects Observed in Plants After Exposure to a Variety of Agricultural Chemicals (Cont'd)

increased or decreased specific gravity of fruit

increased or decreased soluble content of fruit

accumulation of chemicals from the soil

effects produced by some chemicals in plants are detrimental. Indeed, some of the effects that have been noted can be considered as desirable, and the chemical might be exploited to induce these effects for economic purposes. In this regard, the testing of chemicals for effects on plant growth should not necessarily be considered as another obstacle or objectionable feature in its commercial development; but rather should create some excitement in anticipation of serendipitous discoveries of other possible uses for the chemical, since it is rarely possible to predict what effect most chemicals might have on plant life.

Our greatest source of information about the ways in which chemicals affect plants resides in the literature pertaining to those chemicals that are used for pest control purposes, and most specifically those chemicals that are used as herbicides. In spite of much experimental work, particularly with the auxin and photosynthetic inhibiting groups of chemicals, our knowledge about the relationship between molecular structure and herbicidal activity is restricted.

The relationship between chemical constitution and physiological activity has been the subject of intensive study and speculation since the earliest days of organic chemistry. This is not surprising since the initial stimulus to that science came from medicine and from the need to find a wide range of drugs for the cure of diseases. This stimulus remains, but with passage of time and because of rising world population the control of pests in agriculture has also become an urgent problem that has resulted in the development of a massive chemical industry in endeavoring to provide solutions to these problems. The triumphs achieved in some areas of pest control have been many and striking, yet a precise theoretical basis for the design of pesticides still eludes us just as it does in the search for chemotherapeutic agents. An understanding of enzyme and other systems involved in vital processes, and their vulnerability in different organisms is necessary for a more rational development of specific biologically active chemicals. Given such an understanding it would be possible to more readily design chemicals that would interfere with vital systems and if problems of stability and transport in the organism can be surmounted, would provide effective agents needed in the control of diseases and pests.

Almost all commercial herbicides were first developed empirically, and studies of their mode of action were undertaken only after their economic benefits were recognized. The results of these investigations are of considerable interest to biology and agriculture, since they furnish new insights into the physiological processes of plants and at the same time provide some information that is useful toward the rational development of new chemicals to satisfy specific needs.

Although the contrary view is often stated, the current level of biochemical knowledge provides considerable understanding of the primary site of action of many of the herbicides (and other pesticides) in use today. However, information is lacking about the detailed mode of action of phytotoxicants that are thought to act in areas where the fundamental biochemistry of the particular process is obscure, e.g., chemicals that are thought to interfere with cell division, axonal transmission, or growth processes in plants.

The biophysical properties of phytotoxic chemicals have so far attracted much less attention than their biochemical properties. With the exception of those chemicals related to indoleacetic acid (auxins), little is known about their effect on biophysical systems in plants. A proper biophysical organization is necessary for the efficient biochemical functioning of the plant.

In view of the importance of biophysical processes in the economy of plants, it would be very helpful to know to what extent interferences by phytotoxicants with these processes may be responsible for the death of plants. An adverse effect of phytotoxic chemicals on biophysical systems operating in the plant may have a profound effect on its growth and development. Thus, more knowledge is necessary to determine how chemicals affect (1) the osmotic pressure and water uptake of cells, (2) the properties of the cell wall, (3) transpiration and stomatal opening, (4) cell permeability, (5) the uptake of mineral ions, and (6) protoplasmic streaming and the viscosity of the protoplasm.

In a discipline concerned with the physical properties of a living system, particular attention needs to be given to factors of physical environment such as temperature, humidity, light intensity and quality, before and after the experiment. Much of the value of some past investigations is lost because of a failure either to control the environment adequately or to have defined the conditions clearly in the published work. Nonetheless, from the evidence found in the literature, it is unlikely that death of plants caused by interferences of phytotoxic chemicals is due to any large extent to effects on biophysical processes. For this reason subsequent emphasis in this report will be placed on the biochemical effects of chemicals as they relate to phytotoxicity.

It must be realized that the effects of chemicals on plants, whether phytotoxic or stimulatory, are not due to a simple physiological property but may result from many different processes, each physiologically distinct, being affected. For example, one group of chemicals may act in high concentration by osmotic action on living cells; another group may interact at low concentration with the photosynthetic system within green cells; yet a third class of chemicals might by their interaction with the plant's own hormonal system disturb cell elongation or multiplication or their component processes. That there are various physiological processes, either beneficial or detrimental, affected by chemicals in plants is readily emphasized in Table 2.1.

The diversity of principles involved in the action of chemicals on plants precludes unqualified generalizations. For most chemicals, multiple sites and mechanisms of action must be considered a probability along with the possibility that the most sensitive sites differ among species. Many possible sites of action have been described for chemicals used as herbicides, however, their contribution to lethal action may still be uncertain in some cases and unknown in other cases.

Table 2.2 has been included to list the most likely primary mode of action and major function that is modified or disrupted in plants by

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth

1. Inhibit Lipid Synthesis; structural organization disrupted.

Thiocarbamates

S-ethyl diisobutylthiocarbamate	butylate
S-ethyl *N*-ethylthiocyclohexanecarbamate	cycloate
S-(2,3-dichloroallyl) diisopropylthiocarbamate	diallate
S-ethyl dipropylthiocarbamate	EPTC
S-ethyl hexahydro-1*H*-azepine-1-carbothioate	molinate
S-propyl butylethylthiocarbamate	pebulate
S-(2,3,3-trichloroallyl) diisopropylthiocarbamate	triallate
S-propyl dipropylthiocarbamate	vernolate

2. Disrupt Cell Membranes.

Petroleum oils

Bipyridyliums

6,7-dihydrodipyrido [1,2-*a*:2',1'-*c*] pyrazinediium ion	diquat
1,1' -dimethyl-4,4' -bipyridinium ion	paraquat

3. Divert Photosynthetic Electron Transport; energy supply disrupted.

Bipyridyliums

6,7-dihydrodipyrido [1,2-*a*:2',1'-*c*] pyrazinediium ion	diquat
1,1'-dimethyl-4,4'-bipyridinium ion	paraquat

4. Inhibit Enzyme Systems; energy supply disrupted.

Arsenicals

hydroxydimethylarsine oxide	cacodylic acid
disodium methanearsonate	DSMA, (DMA)
monosodium methanearsonate	MSMA

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

Inorganic salts	
ammonium sulphamate	
borax	
calcium cyanamide	
copper sulfate	
sodium chlorate	
sodium metaborate	
potassium cyanate	
Miscellaneous compounds	
allyl alcohol	2-propen-1-ol,
acrolein	2-propenal
5.Uncouple Oxidative Phosphorylation.	
Dinitrophenols	
2-*sec*-butyl-4,5-dinitrophenol	dinitrobutylphenol
2-(1-methylbutyl)-4,6-dinitrophenol	
4,6-dinitro-*o*-cresol	dinitrocresol
Hydroxybenzonitriles	
3,5-dibromo-4-hydroxybenzonitrile	bromoxynil
4-hydroxy-3,5-diiodobenzonitrile	ioxynil
Miscellaneous	
pentachlorophenol	PCP

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

6. Affect Photosynthetic Electron Transport (Hill Reaction Inhibited) and Chlorophyll Destruction via Inhibition of Carotenoid Synthesis;

Ureas

3-(*p*-chlorophenyl)-1-methyl-1-(1-methyl-2-propynyl) urea	buturon
3-(4-bromo-3-chlorophenyl)-1-methoxy-1-methylurea	chlorbromuron
3-[*p*-(*p*-chlorophenoxy)phenyl]-1,1-dimethylurea	chloroxuron
3-cyclooctyl-1,1-dimethylurea	cycluron
3-(3,4-dichlorophenyl)-1,1-dimethylurea	diuron
1,1-dimethyl-3-phenylurea	fenuron
1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl) urea	fluometuron
tert-butylcarbamic acid ester with 3-(*m*-hydroxyphenyl)-1,1-dimethylurea	karbutilate
3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea	linuron
3-(*p*-bromophenyl)-1-methoxy-1-methylurea	metobromuron
3-(*p*-chlorophenyl)-1-methoxy-1-methylurea	monolinuron
3-(*p*-chlorophenyl)-1,1-dimethylurea	monuron
3-(hexahydro-4,7-methanoindan-5-yl)-1,1-dimethylurea	norea
1-(2-methylcyclohexyl)-3-phenylurea	siduron

s-Triazines

2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine	ametryne
2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine	atrazine
2-(ethylamino)-4-(isopropylamino)-6-methoxy-*s*-triazine	atratone
2-[[4-chloro-6-(ethylamino)-*s*-triazin-2-yl amino]-2-methylpropionitrile	cyanazine
2-chloro-4-(cyclopropylamino)-6-(isopropylamino)-*s*-triazine	cyprozine
2-(isopropylamino)-4-(methylamino)-6-(methylthio-*s*-triazine	desmetryne

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

2-(ethylthio)-4,6-bis(isopropylamino)-*s*-triazine	dipropetryn
2-chloro-4-(diethylamino)-6-(isopropylamino)-*s*-triazine	ipazine
2,4-bis(isopropylamino)-6-methoxy-*s*-triazine	prometone
2,4-bis(isopropylamino)-6-(methylthio)-*s*-triazine	prometryne
2-chloro-4,6-bis(isopropylamino)-*s*-triazine	propazine
2-chloro-4,6-bis(ethylamino)-*s*-triazine	simazine
2,4-bis(ethylamino)-6-(methylthio)-*s*-triazine	simetryne
2,4-bis(ethylamino)-6-methoxy-*s*-triazine	simetone
2-(*tert*-butylamino)-4-(ethylamino)-6-(methylthio)-*s*-triazine	terbutryne
2-chloro-4-(diethylamino)-6-(ethylamino)-*s*-triazine	trietazine
Acylanilides	
3',4'-dichlorocyclopropanecarboxanilide	cypromid
N-[5-(2-chloro-1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl]-cyclopropanecarboxamide	
3',4'-dichloropropionanilide	propanil
2-chloro-2',6'-diethyl-*N*-(methoxymethyl)acetanilide	alachlor
Hydroxybenzonitriles	
3,5-dibromo-4-hydroxybenzonitrile	bromoxynil
4-hydroxy-3,5-diiodobenzonitrile	ioxynil
Uracils	
5-bromo-3-*sec*-butyl-6-methyluracil	bromacil
3-cyclohexyl-6,7-dihydro-1*H*-cyclopentapyrimidine-2,4(3*H*,5*H*)-dione	lenacil
3-*tert*-butyl-5-chloro-6-methyluracil	terbacil
Ethers	
p-nitrophenyl α,α,α-trifluoro-2-nitro-*p*-tolyl ether	fluorodifen
2,4-dichlorophenyl-*p*-nitrophenyl ether	nitrofen

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

Pyridazinones	
4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone	monometflurazon
5-amino-4-chloro-2-phenyl-3(2*H*)-pyridazinone	pyrazon
N-phenylcarbamates	
methyl *m*-hydroxycarbanilate *m*-methylcarbanilate	phenmepipham
methyl 3,4-dichlorocarbanilate	swep

7. Inhibit Carotenoid Synthesis; destroy chlorophyll.

3-amino-1,2,4-triazole	amitrole
2,3,5-trichloro-4-pyridinol	pyriclor

8. Inhibit Cellular or Nuclear Division.

N-phenylcarbamates	
4-chloro-2-butynyl *m*-chlorocarbanilate	barban
D-*N*-ethylacetamide carbanilate (ester)	desmedipham
isopropyl *m*-chlorocarbanilate	chlorpropham
isopropyl carbanilate	propham
methyl 3,4-dichlorocarbanilate	swep
Other carbamates	
methyl sulfanilylcarbamate	asulam
2,6-di-*tert*-butyl-*p*-tolyl methylcarbamate	terbutol
Dinitroanilines	
N-butyl-*N*-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine	benefin
N^4,N^4-diethyl-α,α,α-trifluoro-3,5-dinitrotoluene-2,4-diamine	dinitramine
2,6-dinitro-*N*,*N*-dipropylcumidine	isopropalin

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

4-(methylsulfonyl)-2,6-dinitro-*N*,*N*-dipropylaniline	nitralin
3,5-dinitro-N^4,N^4-dipropylsulfanilamide	oryzalin
α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine	trifluralin
Miscellaneous compounds	
6-hydroxy-3(2*H*)-pyridazinone	MH
2-chloro-*N*-isopropylacetanilide	propachlor

9. Mimic Indolacetic Acids.

Phenoxyalkonic acids	
2,4-dichlorophenoxyacetic acid	2,4-D
4-(2,4-dichlorophenoxy)butyric acid	2,4-DB
tris-(2,4-dichlorophenoxyethyl) phosphite and bis (2,4-dichlorophenoxyethyl) phosphite	2,4-DEP
2-(2,4-dichlorophenoxy)propionic acid	dichlorprop
2-(2,4,5-trichlorophenoxy)ethyl 2,2-dichloropropionate	erbon
(2,3,6-trichlorophenyl)acetic acid	fenac
2-(2,4,5-trichlorophenoxy)propionic acid	silvex
[(4-chloro-*o*-tolyl)oxy]acetic acid	MCPA
4-[(4-chloro-*o*-tolyl)oxy]butyric acid	MCPB
2-[4-chloro-*o*-tolyl)oxy]propionic acid	mecoprop
1-naphthaleneacetic acid	NAA
2,4,5-trichlorophenoxyacetic acid	2,4,5-T
Benzoic acids	
3-amino-2,5-dichlorobenzoic acid	amiben
3,6-dichloro-*o*-anisic acid	dicamba
2,3,6-trichlorobenzoic acid	2,3,6-TBA

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

Picolinic acid	
4-amino-3,5,6-trichloropicolinic acid	picloram
10. Interfere with Transport of Indolacetic Acid.	
N-1-naphthylphthalamic acid	NPA
2,3,5-triiodobenzoic acid	TIBA
11. Inhibit Gibberellins; growth and reproduction disrupted.	
2-(chloroethyl)trimethylammonium chloride	chloromequat chloride, CCC
N-(dimethylamino)succinamic acid	succinic acid; DMSA
(2,4-dichlorobenzyl)tributylphosphonium chloride	phosphon
12. Affect Ethylene Levels; growth and reproduction disrupted.	
2-chloroethylphosphonic acid	ethephon
13. May Combine with Proteins.	
Chlorinated aliphatics	
2,2-dichloropropionic acid	dalapon
trichloroacetic acid	TCA
ethylene glycol bis(trichloroacetate)	glytac
14. Mimic Nitrate Ion; disrupt metabolism of nitrogen compounds.	
sodium chlorate	
15. Mode of Action not Known.	
2,6-dichlorobenzonitrile	dichlobenil
O,O-diethyl dithiobis[thioformate]	bisethylxanthogen
1,1,1,3,3,3-hexachloro-2-propanone	HCA
potassium hexafluoroarsenate	hexaflurate

Table 2.2 Mode of Action by Which Various Chemicals Disrupt Plant Growth (Cont'd)

O,O-diisopropyl phosphorodithioate *S*-ester with *N*-(2-mercaptoethyl)benzenesulfonamide	bensulide
3-isopropyl-1*H*-2,1,3-benzothiadiazin-(4)3*H*-one 2,2-dioxide	bentazon
(benzamidooxy)acetic acid	benzadox
N,N-dimethyl-2,2-diphenylacetamide	diphenamid
2-(α-naphthoxy)-*N,N*-diethylpropionamide	napropamide
2-*tert*-butyl-4-(2,4-dichloro-5-isopropoxyphenyl)-Δ^2-1,3,4-oxadiazolin-5-one	oxadiazon

chemicals that are used to control plant life. Over 125 chemical substances ranging from the relatively simple inorganic salts to the more intricate synthetic organic chemicals and complex materials such as petroleum oil are included in this survey. The synthetic organic chemicals are represented by diverse chemical structures consisting of derivatives of acylanilides, alchols, arsenicals, benzoic acid, benzonitriles, bipyridinyls, carbamates, chlorinated aliphatics, chlorophenols, dinitroanilines, dinitrophenols, ethers, methylcarbamates, pyridazionones, sulfonamides, *s*-triazines, triazole, uracil, and ureas.

The available information reveals that death of plants from such chemicals results after they affect such major functions as structural organization, energy supply, and growth and reproduction through at least 12 identified mechanisms of action. Such mechanisms of action listed in Table 2.2 include (1) disruption of lipid synthesis, (2) disturbances of cell membranes, (3) interference with electron transport, (4) inhibition of enzymes, (5) uncoupling of oxidative phosphorylation, (6) inhibition of photosynthesis, (7) inhibition of pigment synthesis and the destruction of chlorophyll, (8) mimicry of plant auxins, (9) interference with plant hormone metabolism, (10) affect level of ethylene, (11) combination with proteins, and (12) interference with mineral metabolism.

The phytotoxic properties of petroleum oils are in direct relationship to the proportion of aromatic constituents they contain. Oils per se probably are not metabolic inhibitors, but act by physically plugging the tissue vessels during transport.

A number of substances such as inorganic salts, chloropicrin, methyl bromide, phenylmercuric acetate, and the substituted chlorophenols are metabolic inhibitors; thus, general poisons that are not specific for plants only. Their effects on plants may be through their established roles of blocking or uncoupling specific enzyme systems, although other unknown toxic actions may be involved.

The best evidence is that arsenic and copper containing chemicals act at least partially by combination or interference with vital thiol groups in the pyruvate dehydrogenase or the α-ketoglutarate dehydrogenase system, or both of them. Pentavalent arsenic may act by mimicking the phosphate ion and be incorporated into key energy containing intermediates that degrade rapidly if they contain arsenate in place of the normal phosphate. In spite of the long usage of ammonium sulfamate, borax, potassium cyanate, and sodium chlorate no satisfactory explanation of their phytotoxic action has been advanced. The general toxic action of calcium cyanamide and sulfuric acid to plants may be ascribed to tissue corrosion on physical contact; although, some as yet unrecognized metabolic action may also be involved.

There is no generally accepted demonstration of any effect of the five types of plant hormones, auxins, gibberellins, ethylene, cytokinins, and abscisic acid, at physiologically realistic concentration in a cell free system. Neither has the mode of action of the synthetic compounds, which affect plant growth, and which are used either as herbicides or as plant growth regulators, been demonstrated with cell free systems.

Corbett (3) postulates that the synthetic growth-regulating chemicals probably act by interfering with the action of the known natural hormones unless there are yet undiscovered natural plant growth hormones that are interfered with by synthetic compounds or if some of the synthetic compounds act at quite different levels than the natural hormones. A likely exception, however, would be those compounds that inhibit cell division. Corbett (3) concludes then that there are only seven ways by which synthetic compounds can interfere with the action of a known natural hormone. It can (1) release or (2) combine with it, (3) interfere with its synthesis or (4) degradation, (5) combine at the site of action, (6) modify its transport or (7) its deposition at an inert site.

The phenoxyalkanoic acids derivatives and related compounds listed under 9 in Table 2.2 acts as auxins. They produce morphological effects on plants that indicate exaggeration of normal auxin action. Hanson and Slife (4) conclude that plants treated with 2,4-dichlorophenoxyacetic acid die due to the aberrant growth produced by its persistent auxin effect. The roots and stem of the treated plant proliferate rapidly, thus appropriating most of the available food, which results in senescence and physiological malfunction of leaves and secondary roots, so that the plant is unable to feed itself, and death ensues. Existing reports in the literature offer ample evidence to rule out both interference with oxidative phosphorylation in mitochondria and inhibition of the Hill reaction in chloroplasts as mechanisms to explain the phytotoxic action of the phenoxyalkanoic acid type of chemicals.

The phenoxyalkanoic acid chemicals are eminently more phytotoxic to dicotyledons than to monocotyledons, generally killing the former but not the latter. There are striking exceptions, however since onions (monocotyledons) are susceptible to these chemicals, where as chickweed and cleavers (Goose Grass-dicotyledons) are resistant.

Although 4-amino-3,5,6-trichloropicolinic acid (picloram) is structurally distinct from the other auxins, it has been shown to possess the typical properties of an auxin. Thus, it causes cell elongation in a variety of plant stems, stem proliferation, leaf and stem bending, loss of chlorophyll, and adventitious rooting.

Derivatives of benzoic acid act in the same way as the phenoxyalkanoic acids, i.e., they are persistent auxins, with the exception of 2,3,5-triiodobenzoic acid (TIBA) which is not an active auxin. TIBA is known to exert its phytotoxic effects by way of interfering with auxin transport, and possibly by modifying auxin action in some other manner. A similar mode of action has been identified for the naphthyl-phthalamic acid and chloroflurecol derivatives.

The chemicals listed under 9 and 12 in Table 2.2 affect plants by reducing growth without permanently stunting or malforming the plant, and their action is distinguished from that of such growth inhibitors as maleic hydrazide. These chemicals are in contrast with almost all of the others listed in Table 2.2. Whereas the latter group are primarily used for their phytotoxic properties to kill plants, the naphthyl-phthalamic acids and chloroflurecols produce compact plants with shortened internodes and leaf stems and are used commercially for such purposes to prevent lodging of cereal stems, increase resistance to insect and fungal attack, control vegetative growth of fruit trees, and to modify the shape and height of ornamental plants.

2-Chloroethylphosphonic acid (ethephon) acts in plants by decomposing to ethylene, thus produce all of the characteristic responses in plants attributed to ethylene. The chemical, therefore, finds practical application to induce flowering, promote fruit ripening, and fruit abscission.

The *N*-phenyl carbamic acid derivatives (Table 2.2.8) affect cell division in plants. They are not used as growth regulators, as are the chemicals mentioned in the preceding paragraph, but rather find extensive application as herbicides, and in this regard are referred to as "mitotic poisons." The inhibitory effect of the *N*-phenylcarbamates on cell division appears to be due to disorientation of the microtubules while other subcellular structures are unaffected. Microtubules are small tube-like structures made of sub-units of globular protein, which may function to aid in the separation and alignment of chromosomes during nuclear division. The action of these chemicals is in contrast to colchicine, a compound known for its ability to inhibit cell division which actually disrupts the microtubules, probably by binding to a component protein. Isopropyl carbanilate (propham), an *N*-phenylcarbamate, has been shown to have no deleterious effect on the tubules themselves, but only on their organization. Morphological and cytological investigations indicate a similar mode of action for all carbamates.

Moreland and Hill (5) concluded that carbamates interfered with more than one system in plants, and produced evidence to show that photosynthesis might be such a system. It is uncertain whether the inhibition of photosynthesis is a primary effect of this class of chemicals. In vitro results strongly suggest that the inhibition of photosynthesis can only be a side effect for most of these chemicals. The *N*-phenylcarbamates that inhibit photosynthesis are structurally similar to the phenylurea and acylanilide derivatives (Table 2.2, 6), chemicals are known as potent inhibitors of photosynthesis, but their toxic symptoms differ and the implication on inhibited photosynthesis to overall herbicidal action is uncertain. Most of them inhibit cell division in both susceptible and resistant plant species.

The benzenesulfonyl and *N*-methyl carbamate derivatives appear to exert their phytotoxic activity also by inhibiting cell division. The dinitroaniline compounds (Table 2.2,8) are analogous in their mode of action to the carbamate class of chemicals. It is known that the dinitroanilines inhibit mitosis, their primary mode of phytotoxic activity. The exact mechanism of how they disrupt nuclear and cell division is not known. The most typical symptoms of plants treated with dinitroaniline chemicals is the inhibition of lateral root formation. They also cause growth reduction in roots and shoots, and swelling and irregularities in various tissues. This class of chemicals is more toxic to monocotyledons than to dicotyledons.

The effect of maleic hydrazide on plants is due to its ability to inhibit cell division, but not cell enlargement. It is an isomer of uracil, a pyrimidine found in ribonucleic acid. It may interfere with mitosis by becoming incorporated into ribonucleic acid, as recent studies have demonstrated.

Herbicides that are toxic to plants by interfering with photosynthesis do so by two distinct modes of action. The majority of the chemicals, for which there is experimental evidence, do so by inhibiting electron transfer, but other compounds such as the bipyridylium derivatives short circuit electron transfer and generate toxic molecular species.

A wide variety of chemicals (Table 2.2, 6) will inhibit photosynthetic electron transfer, and many of them are used as herbicides. The urea and *s*-triazine family have more such herbicides than any other class of chemicals. More is known about the mode of action of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) than for any other inhibitor of the Hill reaction. The studies with diuron have contributed significantly to our understanding of the mechamisms of photosynthesis and herbicidal properties of such chemicals. Only a limited amount of biochemical research has been reported for ureas other than diuron and 3-(4-chlorophenyl)-1,1-dimethylurea (monuron); however, the available data suggest that all urea herbicides inhibit the Hill reaction and thus appear to have similar modes of phytotoxic action.

The phototoxic action of the substituted urea chemicals appears to be due to other factors as well as the inhibition of photosynthesis. Diuron, for example, affects some non-photosynthetic processes, but only at very high concentrations. Death of plants caused by this class of chemicals is probably due to the direct interruption of the food supply caused by inhibition of photosynthesis, and by irreversible damage to the photosynthetic system, resulting in permanent inhibition of food supply.

The *s*-triazine class of chemicals are also potent inhibitors of the Hill reaction, thus their primary mode of action is due to the inhibition of photosynthesis. The available information suggests that they act in an essentially similar way to the substituted urea compounds and kill plants in a similar but not necessarily identical manner as the ureas.

The acylanailides have been studied much less than the ureas and the *s*-triazines, however, they also have proved to be powerful inhibitors of the Hill reaction. The available evidence suggests that they act in a similar manner as the ureas and *s*-triazines. The hydroxybenzonitriles are both uncouplers of oxidative phosphorylation and inhibitors of photosynthesis. It is not possible to be conclusive about which of these effects is most important in the phytotoxic action. The fact that light is necessary to achieve rapid and complete killing of plants with some of the hydroxybenzonitrile compounds indicates that the herbicidal action of these chemicals is mainly due to inhibition of photosynthesis.

The uracil derivatives cited in Table 2.2 inhibit the Hill reaction and are regarded to exert phytotoxic effects by their inhibitory action on photosynthesis. They appear to have little effect on non-photosynthetic processes in plants. The available information about the uracil compounds reveals that they act in a similar fashion to the ureas. Some of the uracil derivatives have been shown to inhibit nitrate reductase activity in excised plant tissue, but the significance of these observations to the phytotoxic action of these chemicals is obscure.

Some of the nitrophenyl ethers have been found to inhibit the Hill reaction and to be active uncouplers of electron transport. Either of these inhibitory effects might be sufficient to explain their phytotoxic action, but there is not sufficient information in the literature that permits one to decide whether the inhibitory effect on photosynthesis or respiration is the primary mode of action in whole plants. The pyridazinones and pyrimidones are also strong inhibitors of the Hill reaction and exert their phytotoxic activity by inhibiting the photosynthetic processes in plants. Other classes of organic chemicals that have been found to be effective inhibitors of the Hill reaction include substituted benzimidazoles, imidazopyridines, imidazoles, imidazoquinoxalines, and thiadiazoles. The benzimidazoles and the imidazopyridines are also uncouplers of oxidative and photophosphorylation. None of these chemicals, however, has been developed for weed control purposes.

The mode of action of the chlorinated aliphatic acids is not known, but it has been suggested that they act by combination with proteins.

Both trichloroacetic acid (TCA) and 2,2-dichloropropionic acid (dalapon) have been shown to inhibit the synthesis of the surface layer of wax on plant leaves, but it is not known whether or not the inhibition of lipid synthesis contributes to the lethal effect of these chemicals after they are applied to plants. Lisner (6) states that dalapon is a specific blocking agent in the biosynthesis of pantothenic acid in plants. Thus, plants with a low requirement for this vitamin might be expected to be resistant to dalapon.

The extensive literature on the possible mode of action of 3-amino-1,2,4-triazole (amitrole) suggests that it might exert its phytotoxic effect by inhibiting more than one reaction in plants. This evidence suggests that amitrole prevents the synthesis of a component that is required for the production of chlorophyll, but does not affect the synthesis of chlorophyll itself. Since, as it has been stated previously, there is reasonable evidence that carotenoids are necessary to prevent the photooxidation of chlorophyll and the disruption of chloroplast structure it seems likely that the inhibition of carotenoid biosynthesis is the primary site of action of amitrole.

On the preceding pages many possible sites and mechanisms of phytotoxic action have been cited for a wide array of chemicals. These findings are a result of the great advances in the development of many new methods to test biochemical responses in in vitro and in vivo systems. Consequently, it is logical to question whether or not such tests could be utilized to evaluate chemicals for potential effects to plant life.

Photosynthesis is the ultimate source of all energy used in plants (and animals) and the toxic action of the largest number of chemicals listed in Table 2.2 have been attributed to their effects on this biochemical process. Therefore, it is obvious that this might be the first useful evaluation to be performed to screen a chemical for possible phytotoxic effects.

There are a number of relatively simple tests for determining the effects of a chemical on photosynthesis. Most of these are described in detail by Wilkinson (7). The Hill reaction, which was mentioned most often in the preceding pages, is a rapid and sensitive spectrophotometric assay by which the photolysis of water by a cell free preparation of photosynthetic tissue can be studied. The usefulness of this test, however, is greater in studying the mechanism of action of a chemical more so than in predicting whether or not a chemical will be toxic to a plant. This is because not all chemicals that effectively inhibit the Hill reaction are phytotoxic when tested on whole plant systems. The converse of this, however, has also been demonstrated, i.e., all chemicals whose mechanism of action has been determined to be due to inhibition of photosynthesis are also inhibitors of the Hill reaction. By way of illustration, all of the herbicidally active *s*-triazines yield a 50% inhibition response of the Hill reaction in concentrations of $10^{-4}$to 10^{-7}M. Although there is generally good agreement between the herbicidal activity and the concentration of the chemical necessary to inhibit the Hill reaction, this relationship is not always consistent. For example, 2-chloro-4-ethylamino-6-*n*-butylamino-*s*-triazine is one of the most active members of this class of chemicals as measured by the Hill reaction, yet it is virtually devoid of herbicidal properties on growing plants in a field.

From the preceding example it is apparent that the interference of a fundamental physiological process, as determined by in vitro testing, does not automatically lead to a similar effect in whole organisms. The reason for this is due to some additional and specific morphological, physiological or biochemical properties of plants and edaphic factors that may influence the stability, fate and behavior of the chemical in soil; hence its availability to the plant.

The diversity of principles involved in phytotoxic activity precludes unqualified generalizations about mechanisms. For most chemicals multiple sites and mechanisms of action must be considered a probability along with the possibility that the most sensitive sites differ among plant species. Many possible sites of action have been described, but their contribution to lethal action is uncertain. The most difficult aspect of mechanism research on phytotoxicity is substantiating the physiological significance of in vitro results under field conditions. At the present state of knowledge in the field of plant physiology this appears to be equally true for whatever biochemical or in vitro parameter that is being considered, i.e., effects on respiration (CO_2 production, O_2 consumption, or high energy phosphate, ATP production); photosynthesis (O_2 release, CO_2 incorporation, chlorophyll content, photophosphorylation, NADP reduction, $^{14}CO_2$ fixation, or the Hill reaction); amino acid and protein metabolism; plant enzyme systems; lipid synthesis; mineral metabolism; and plant nucleic acids. The same would also be applicable to morphological, anatomical, and cytological studies.

The susceptibility of a plant to a particular chemical is influenced by morphological, physiological, biochemical, and genetic factors. These factors influence the concentration of material that reaches the site or sites of action at any one time. The site(s) of action in different species of plants may also be differentially sensitive to the chemical.

The quantity of a chemical accumulated per unit weight of plant may be important, since as a rule the higher the concentration of chemical in the plant the more likely it is to damage it. The difference in the degree of absorption can be caused by the morphological characteristics of the plant in question. After a chemical is absorbed by a plant it must be translocated to the site of action and not remain at the site of its application. While a study of the morphological attributes of plants often allows one to predict the characteristics necessary for penetration of a chemical, similar predictions cannot always be made with regard to the physiological response of the plant. Experiments alone can decide whether a particular chemical is more toxic to one species than to another.

The differences in the responses of the same dose of chemicals to different plant species has been the basis of much research. A number of factors may operate in determining the selectivity of this response, amongst the important ones are (1) the extent to which the applied chemical is absorbed by the plant, (2) the ease with which the chemical moves in the plant tissue, (3) the stability of the chemical within the plant, and (4) its potential activity at the site of action. A chemical may be applied in one form, translocated in a metabolized form, and express its inhibitory effect at the site of action in still another form; whereas, another chemical may be absorbed, translocated, and accumulated at the site of action in its native state. Almost all organic chemicals are metabolized by plants, many rapidly and extensively.

Metabolism of a chemical is not necessarily tantamount to detoxification, although this is usually the case. There are a number of instances in which a relatively non-toxic chemical is converted by plant metabolism to phytotoxic material, e.g., reduction of dipyridyl compounds and the β-oxidation or ester hydrolysis of some of the phenoxy compounds.

The problem of extrapolating in vitro tests to in vivo conditions is confounded also by the problem of secondary effects. Despite our reasonably good knowledge of the primary sites of action of many chemicals, generally very little is known about the secondary reactions resulting from the initial action of the chemical.

The complexity of methodology and high cost are also additional deterrents to the use of biochemical and other laboratory tests for studying the effects of a chemical on plant life. A number of biochemical tests such as the Hill reaction and other tests for photosynthesis and respiratory activity are relatively simple and can be conducted by most competent laboratory technicians with equipment available in any adequately equipped chemical laboratory.

However, many other biochemical testing procedures require a considerable amount of costly and specialized equipment and supplies as well as highly skilled personnel not usually found in laboratories that are not normally involved with biochemical operations. The degree of specialization and expertise becomes even increasingly greater for cytological investigations.

It is therefore concluded that for the present it is not feasible to employ biochemical test methods to obtain quantitative estimations of the phytotoxic properties of chemicals that could be correlated in any meaningful way with the actual behavior of the chemical under field conditions. In view of our present knowledge of primary biochemical disturbances that can be caused by chemicals in plants, the complexity, high cost, and requirements for highly specialized laboratory equipment and personnel, the phytotoxic properties of chemicals can best be compared on relatively simple criteria based on numbers of plant individuals damaged or destroyed in bioassay type tests.

A very large amount of time was spent during an intensive effort to consider the practicality of using biochemical test methods to elucidate the potential phytotoxic properties of chemicals. The results of this effort have been negative in respect to adapting such procedures on a practical basis at the present. However, the effort expended revealed that the present state-of-the-art is somewhat incoherent, in disarray, but excitingly evolutionary. The writer would be remiss, therefore, if he neglected to recommend that this specific phase of the present contract be considered for further in depth consideration. There appears to be a need for an intensive study to compile and to correlate the vast amount of available information regarding the biochemical responses of plants to chemicals, with the expected resultant effect that a number of existing procedures might already be available to establish a better relationship between chemical structure and physiological activity, and to cite new areas of research that should be undertaken to provide presently missing answers for more practical utilization of biochemical test methods in the field of phytotoxicity.

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2.3 FATE OF TOXICANTS IN OR ON PLANTS

The fate of any compound contacting a plant may follow several pathways. In some instances this may result in degradation and finally the material's passing from existence. In other instances the compound may remain intact and merely recycle in the environment. The alternatives have been outlined by Norris in a paper presented at the Environmental and Physiologic Chemodynamics Symposium at Oregon State University in 1969. The following discussion is based on that paper.

The initial point of chemical-plant contact depends on the method of release. In some instances the foliage and stems may be the primary intercepting organs. In other instances roots or rhizomes may be the initial contact.

2.3.1 Aerial Portions of the Plant

Materials intercepted by aerial portions of the plant may undergo several processes:

2.3.1.1 Absorption. The degree of absorption will dictate the severity of effects with systemic chemicals. The amount of absorption depends on the nature of the chemical, the plant species and the chemical residue characteristics. Absorption must take place if toxicity to the plant is to occur.

2.3.1.2 Surface adsorption. The extent of adsorption depends on the physical and chemical properties of both the chemical and the leaf surface. Surface adsorption may inactivate chemicals since it prevents absorption and reduces contact action. It is important to realize, however, that surface adsorption is not final; it is an equilibrium reaction. Environmental factors define the equilibrium between adsorbed and free chemicals and a change in environmental conditions will alter the point of equilibrium. Any reduction in the amount of free chemical leads to a release of adsorbed chemical until equilibrium is reestablished.

2.3.1.3 Volatilization. This process is not important for chemicals with a low vapor pressure or a high heat of vaporization. On the other hand, losses may be appreciable for pesticide compounds like ethyl *N*,*N*-dipropylthiolcarbamate (EPTC) or the isopropyl ester of 2,4-D. Although volatilization reduces chemical residues on the plant, it adds to the total load of atmospheric pollutants.

2.3.1.4 Washoff. The amount of chemical not absorbed, adsorbed, degraded, or lost through volatilization may be subjected to washoff. Washoff may carry materials in solution or suspension depending on their water solubilities. Chemicals washed to the soil may be leached to the root zone and absorbed by the plant.

2.3.1.5 Degradation. Degradation of surface residues may reduce activity by removing the chemical from the site of action. On the other hand, degradation is the only mechanism which can reduce the total load of environmental pollutants. Absorption, adsorption, volatilization, and washoff only store or transport hazardous substances to other parts of the environment.

2.3.2 Underground Portions of the Plant

Chemicals in the root zone are subjected to the same processes as chemicals intercepted by aerial portions of the plant. However, the degree to which a particular process operates may be quite different.

Water-soluble materials are readily absorbed by the roots and may be transported to other parts of the plant. Surface adsorption also occurs. Volatilization is relatively unimportant from root surfaces, but may occur from the soil surface. Washoff does not occur, but leaching of chemicals from the root zone is an analogous process. Photochemical degradation does not occur on roots, but chemical and biological degradation in the root zone is important.

2.3.3 Behavior of Substances Inside the Plant

The action of chemicals inside the plant depends on absorption and activity. If significant amounts are absorbed, the substance may have profound physiologic effects on plant growth. Chemicals inside plants may undergo several processes:

2.3.3.1 Translocation. Translocation is important because the fate of chemicals may vary in different plant parts. Materials absorbed by foliage but not translocated to other plant parts may be lost in leaf fall, while those transported to the roots may be exuded into the soil. Generally, mobility and water solubility are positively correlated.

2.3.3.2 Storage. The chemical or physical binding of substances to plant constituents, may occur in any part of the plant. Largest amounts are frequently found close to the point of absorption, in storage cells adjacent to the paths of translocation, and in areas of intense metabolic activity. Storage may be active or passive. Active storage is accumulated against a concentration gradient and requires expenditure of metabolic energy. Chemicals may be passively adsorbed to structural components of plants. Both active and passive storage are reversible, and materials may be released and translocated to other parts of the plant as conditions in the plant change.

2.3.3.3. Metabolism. Alteration in chemical structure may result in detoxication or activation and may occur anywhere in the plant. Metabolism of most chemicals is nearly always a detoxication process for the plant, but the products may be biologically active in other systems and, therefore, still important as residues. The phenoxybutyric herbicides are an exception. They are inactive as herbicides, but their herbicidally active acetic acid derivatives are produced through β-oxidation of the butyric side chain.

2.3.3.4 Exudation. Volatile substances and metabolites may leave the plant as vapors through the stomates (pores in the leaves). Some chemical herbicides like 2,4-D, 2-methoxy-3,6-dichlorobenzoic acid (dicamba) or 4-amino-3,5,6-trichloropicolinic acid (picloram) are exuded from the roots. In contrast with animals, fish, and birds, however, exudation of chemicals from plants is not extensive.

2.3.4 Conclusions

The chemical characteristics of a substance determines its fate in all parts of the environment including plants. Chemicals in plants may be absorbed, stored, metabolized, and/or recycled to the environment. These processes determine both the substance's impact on the plant and its residue characteristics.

The behavior of a chemical results from the interaction between the properties of the compound and the environment. The environment has many components, and a chemical may interact with any or all of them. The chemical behavior we observe in a pollutant in nature is an integration of many single interaction.

Physiologists can accurately measure both the chemical properties and the environmental factors which interact to produce behavior. The results of some simple interactions can be predicted. However, the field of chemodynamics has not yet attained the sophistication necessary to quantify the multiple interactions which may occur between chemicals and their environment.

It will be impossible to derive a single predictive test which includes the important primary and secondary interactions which produce chemical behavior and effects within the plant.

2.4 CRITERIA FOR SELECTION OF TEST METHODS

Choice of protocols for evaluation of substances for physiologic activity depends on the following factors:

1. Chemical configuration of compound under study
2. Geographic location of entry site(s) into environment
3. Type of entry
    a. Unintentional direct emission - from manufacturing or processing facilities
    b. Intentional release - for intended use within natural environment
    c. Indirect release - from various products, endproducts or components containing the questionable substance
4. Concentration of compound at entry
    a. Weight of compound per weight of transport or contact medium
    b. Total quantity of release
5. Phase of compound at entry
    a. Solid
    b. Vapor
    c. Liquid
        (1) Solution
        (2) Suspension
6. Site of plant interception
    a. Aerial portion
        (1) Foliage

        (2) Support structure

        (3) Fruit

    b. Underground portion

        (1) Roots

        (2) Rhizomes, tubers, etc.

Selection of a test procedure can be made on a much sounder basis if information about the preceding factors is available at the time of testing. Direct emission of a particulate substance through air control system of a manufacturing facility near intensive vegetable crop acreages represents a completely different situation from liquid effluent released into a waste water stream used for irrigation of forest land.

Knowledge of chemical stability or persistance is important. Will the material hydrolyze rapidly and degrade after initial entry to the environment? In this instance the potential for plant damage may be present only at the time of release and in the vicinity of first contact. On the other hand a stable molecule may remain active in soils and waters and even recycle in the plant-to-plant system, affecting future generations of crops and/or native vegetation or plant communities.

Ideally, a test procedure should evaluate a complete life cycle or generation, i.e. seed to seed or spore to spore. Unfortunately, for most materials, this is not possible. Resources are not available to carry out such procedures. In the case of woody perennials, the time involved, especially in community population studies, could span a human lifetime or more.

Chemical evaluation and screening procedures have been used for over twenty-five years with the goal of seeking physiologic activity for refinement herbicides and/or plant growth regulators. Much has been learned from this activity within the plant science research disciplines. The procedures may be grouped in the following way:

2.4.1 Scope or Extent of Procedure

1. Seed germination (petri dishes)
    a. incubator
    b. growth chamber
2. Seedling evaluation
    a. incubator
    b. growth chamber
3. Growth effects (seedling or transplants)
    a. liquid medium
    b. solid medium
4. Growth effects (complete life cycle)
    a. pots
    b. field plots
5. Species shifts
    a. synthesized communities
    b. natural communities
6. Population shifts
    a. synthesized communities
    b. natural communities

2.4.2 Facilities for Test Procedures

1. Laboratory

2. Incubator
3. Growth chamber
4. Greenhouse
5. Field plots
6. Native vegetation areas

2.4.3 Treatment Methods for Test Substance

1. Seed treatment--(soak, dust, etc.)
2. Other germination substrate, e.g. filter paper, (dip, soak, etc.)
3. Seedling foliage spray or growth medium amendment
4. Soil amendment, pre-plant
5. Soil drench - post plant
6. Soil vapor transfer, (volatilization chamber)
7. Mature plant foliage spray
8. Field soil spray
    a. pre-emergence
    b. post-emergence
9. Field soil granular incorporation (pre-plant)
10. Exposure to atmospheric gas or aerosal
11. Field spray or granules (native plant communities)

2.4.4 Evaluation of Factors Influencing Plant Response and Chemical Activity

1. Chemical treatment procedure
    a. Spray or drench
        (1) spray droplet size and pressure
        (2) chemical dilution and/or dosage
        (3) wetting agents

        - (4) spray volume per plant or unit of growth
    - b. Soil amendment
        - (1) granule or dust particle size
    - c. Germination substrate, e.g. filter paper
        - (1) incorporation method
        - (2) solvent choice
- 2. Pre-treatment plant environment
    - a. Above ground
        - (1) temperature
        - (2) humidity
        - (3) light intensity
        - (4) photoperiod
    - b. Below ground
        - (1) soil moisture
        - (2) soil temperature
        - (3) pH of soil solution
        - (4) soil texture
        - (5) organic matter
        - (6) nutrient element balance
    - c. Chemical residue persistance and mobility in soil
- 3. Treatment plant environment
    - a. Above ground
        - (1) temperature
        - (2) humidity
        - (3) light intensity
        - (4) photoperiod

- b. Below ground
  - (1) soil moisture
  - (2) soil temperature
  - (3) pH of soil solution
  - (4) soil texture
  - (5) organic matter
  - (6) nutrient element balance
- c. Chemical residue persistance and mobility in soil

4. Post-treatment plant environment
   - a. Above ground
     - (1) temperature
     - (2) humidity
     - (3) light intensity
     - (4) photoperiod
   - b. Below ground
     - (1) soil moisture
     - (2) soil temperature
     - (3) pH of soil solution
     - (4) soil texture
     - (5) organic matter
     - (6) nutrient element balance
   - c. Chemical residue persistance and mobility in soil

2.4.5 Plant Age at Treatment and Exposure Duration

1. Seed germination
2. Seedling

3. Mature plant

4. Complete life cycle

5. Complete life cycle in ecosystem-community situation

2.4.6 Evaluation Criteria for Chemical Treatment (Most tissues and organs have been employed)

1. Size

2. Weight

3. Morphology

4. Crop yield

5. Reproduction

6. Competition and survival in natural communities

3. RECOMMENDED TEST PROTOCOLS

The difficulty in predicting toxicity from a single or even a limited number of test procedures has been repeatedly emphasized. However, any evaluation program must have a beginning. In view of this situation a sequence of three levels of testing beginning with laboratory-growth chamber (seed germination-seedling effects), greenhouse and extending to open plot has been recommended. These procedures are not intended to be adhered to rigidly but rather to serve as framework for further development and procedural modification as the needs may arise. In addition, a procedure is provided for bacterial nitrogen-cycle and fungal cellulose decomposition inhibition testing.

These procedures have been derived from the relevant procedures cited as well as from interviews and the knowledge of the consultants. In the later stages of testing, special attention should be given to the listing of references involving environmental effects and interactions, such as soil temperature, pH, moisture, texture, air environment, and chemical interactions. The need to move to greenhouse and field testing depends on the anticipated amount of environmental release of the substance.

The fate and effects of many chemicals or their degradation products are of concern because such chemicals enter the soil through the various manners by which they are used. Many are applied as pesticides directly to the soil to selectively control soil insects, fungi, nematodes or weeds, or, to be taken up from the soil by plant roots and translocated into their aerial portions to protect them by means of systemic action from bacteria, fungi, insects and mites, or, to unselectively kill vegetation on industrial sites. Still other pesticides reach the soil as "fallout" or drift after spray applications to the aerial parts of plants or find their way into the soil as a result of the decay of plants that have been treated with them.

Apart from the desired toxic effect of the pesticide on the target organism there are a number of possible secondary effects that they may have on non-target organisms. Some of these chemicals, especially those used as herbicides, may persist in the soil for a long period, thus damaging or reducing the yield of sensitive crops subsequently grown in treated soils. The chemicals may also leach into drainage water, ultimately resulting in a potential danger to man, animals, and plants through the contamination of potable and irrigational water. Yet another very important possibility is the direct action of such chemicals on one or more of the components of the complex microbial population in the soil. Soil fertility depends on a very delicate equilibrium being maintained in this population. Its disturbance by incident chemicals might indeed affect fertility adversely.

It is of concern, therefore, to determine whether or not chemicals in the soil may cause undesirable changes in the microbial populations or in the metabolism of the microbial community. Such changes could affect the development and vigor of plants through nutritional disturbances, a shift in biological equilibrium, or the appearance of microbial inhibitors for plant growth. Chemicals entering the soil in large amounts, at frequent intervals, or those that do not detoxify rapidly could conceivably eliminate or suppress microbial groups with a subsequent reduction in crop yield or quality.

Considerable investigative attention has been directed toward studying the influence of pesticides on biological process or on the development of specific microorganisms in the soil. Excellent reviews by Audus (1, 2), Alexander (3), Martin (4), Domsch (5), Bollen (6), and Fletcher (7) have covered the subject extensively.

Bioassay tests have been developed to assess the toxicity of pesticides to microorganisms by observing their effects on the rate of growth of selected species, by comparing the numbers of soil microorganisms capable of growing in media containing various rates of the chemical, and by noting the rates of changes in specific biochemical processes in enrichment cultures fortified with various rates of the pesticide. Respiration, nitrogen mineralization, rate of nitrification, and cellulose decomposition are the biochemical processes most commonly considered for this purpose. No one of these techniques is entirely suitable to characterize the influence of the added chemical and some provide little information of ecological significance. However, an insight into the possible alterations of the soil microflora can be obtained by judicious use of several of the procedures.

Herbicides are applied to soil at very low rates (several parts per million) and at these rates most of them appear not to affect the dominant microbial groups. Tenfold or higher concentrations of herbicides than those employed for recommended weed control purposes are usually required before inhibitions are noted. A few of the herbicides studied, however, have been observed to cause extended depressions of microbial numbers. Dinoseb, at low rates, can reduce the overall bacterial population for as long as three months (8). Dalapon and EPTC, at normal field rates, reduced total bacterial and actinomycete numbers for approximately two months, although nitrifying organisms were unaffected (9). Fungal populations were depressed at normal or just above normal field rates by dinoseb and pyrazon in calcareous soil (10). Fitzgerald (11) reported that algae are sensitive to low levels of monuron.

Insecticides are often applied to or reach the soil as "fallout" in appreciably higher concentrations than herbicides. However, reasonably large quantities are required before significant inhibitory effects are observed. Partial inhibition of one or another microbial group has been reported in certain soils with many insecticides, but these changes are rarely dramatic (12).

Fungicides and soil fumigants have a much greater effect on microbial populations than herbicides or insecticides. In general, fungicides at normal rates augment bacterial numbers, presumably in an indirect manner by killing fungi, thus increasing the organic substrates for bacterial growth, and reducing the level of antibiotic substances produced by these fungi (2).

Many fungicides are broad spectrum toxicants and can serve as partial soil sterilants, killing large segments of the saprophytic population. The microbial response to these fungicides and fumigants can be divided into four phases (3). In the initial phase, populations of microorganisms commonly decrease and may remain low for long periods. Fewer fungi have been found in some soils fumigated with D-D than in parallel untreated plots even three years after treatment (13). The second stage, lasting for shorter periods of time of up to eight months or so, includes the multiplication of a variety of microbial types which often attain cell densities in excess of those in the original unamended soil. In the third phase, a new biological equilibrium frequently is established as a result of the pesticide application. The composition of the microflora is less diverse as a result of treatment and often one, two or more species assume dominance. The final phase is essentially the climax community characteristic of the original soil. These oscillations in microbial numbers probably follow the rise and fall of available nutrients from autolysing organisms that have been killed by the fungicide or from the added fungicide itself. Antagonistic effects between specific groups of microorganisms probably play a role in these oscillations.

The organisms which decompose the various forms of cellulose are some of the most important microorganisms contributing to the humification processes in soils. The effects of pesticides on these processes have been studied mainly by direct observations of changes in populations of cellulolytic organisms, and also by following the decomposition of cellulosic substrates buried in treated soils (2). A few herbicides when applied at normal field rates have shown significant inhibition of cellulolytic bacteria for relatively short intervals, e.g., fenuron, atrazine, simazine, and propazine (2). Most of the insecticides tested have no effects on the numbers of activities of cellulolytic organisms in soil at normal field rates. Knowledge is fragmentary on the effects of fungicides and fumigants on cellulolysis in soils. Methyl bromide and D-D at normal rates completely suppress activity for days while ethylene dibromide is only inhibitory at very high rates (2).

Most herbicides fail to inhibit the rates of respiration, organic matter turnover, and nitrification in the soil at normal field rates. Nonsymbiotic nitrogen fixation, nitrogen mineralization, and ammonification usually show no response to soil treatment with herbicides. Nitrification is one of the most sensitive conversions in the soil being inhibited by concentrations of chemicals not inhibiting other important biochemical reactions. Quantities of herbicides required for a significant decline in the rate of nitrate formation are often appreciable, e.g., 12 ppm CDAA, CDEC, or CIPC, 10 ppm DNEP or 25 ppm of monuron.

In general, insecticides have no effects on the CO_2 production, cellulose decomposition, ammonification, and nitrification process in the soil except at relatively high dose rates (14, 15). Nitrification and nodulation of leguminous plants have been shown to be inhibited by some insecticides, but the effects are not consistent from soil and rarely is the toxicity complete (3).

Fungicides and fumigants, when applied at normal field rates, are the compounds that most often dramatically alter the biological activity in soil. Nitrification has been found to be inhibited by a number of fungicides and fumigants. Following treatment, the typical pattern consists of a strong suppression of the nitrifying microorganisms followed by a slow recovery as shown by methyl bromide (recovery several weeks or more), D-D (recovery 50 days or more), chloropicrin (recovery 50 to 120 days), nabam (recovery 60 days), thiram (recovery 60 days), ferbam (recovery 28 days), maneb (recovery 25 days), zineb (recovery 17 days), mylon (recovery more than 60 days), and CS_2 (recovery over five months) (2). Temporary suppression of nitrate biosynthesis can be beneficial in as much as the nitrogen is not lost from the plant's rooting zone by leaching or denitrification; however, ammonium accumulation arising from the lack of nitrifying activity in treated soils may lead to serious root injury (3). Also destruction of the nitrifying bacteria may be responsible for a reduction in plant growth or crop yield because of lack of nitrate availability. Morris and Gibbons (16) reported that nitrate fertilizers were especially beneficial to tobacco growing in a soil in which the nitrifiers had been killed as a consequence of fumigation.

Differential effects of the microscopic species in the soil is part of the general pattern of pesticide action. Toxicity varies with the individual microbial species, the morphological and physiological stage of the organism (endospore, conidia, sclerotia, vegetative cells, hyphae, etc.), the chemical and physical composition of the environment, and the time of exposure of the organism to the toxicant (e.g., the age of the organism) (3).

Kreutzer (17) reviewed many instances of selective toxicity of chemicals to soil microorganisms. In general, the sensitive species are reduced in abundance, and their processes retarded. The tolerant species, and their processes, assume dominance as a result of the elimination of competing species. Major emphasis has been placed on the selective effects of pesticides on soil fungi, especially the plant pathogenic fungi. Alexander (3) states that chemicals can alter the populations of a plant pathogen in three ways: (1) they may exert a direct destructive action upon the pathogen; (2) they may have no direct eradicating influence upon the pathogen, but rather the chemical might destroy or reduce the number of the pathogen's natural antagonists, resulting in a rise in the abundance of the pathogen and/or an increase in the severity of the disease it incites; and (3) they might destroy the organisms which serve to control the pathogen's antagonists so that the antagonists become more numerous and suppress the pathogen.

The selection of antagonistic species following the treatment of a soil with a specific chemical may occur because of a selective destruction of components of the indigenous population or because of the rapid rate of recolonization of the antagonists following partial soil sterilization. Strains of the genus *Trichoderma* are considered important antagonists since they suppress *Armillaria*, *Pythium*, *Rhizoctonia*, *Phytophthora*, and other soil-borne pathogens (3). Of special significance is the fact that *T. viride* is frequently found to be the dominant fungus following the application of toxic chemicals to soil (18,19). Its dominance appears to be due to its high rate of growth as much as to its tolerance of the chemical (6).

These same selective phenomena could also account for the abundance of increased activity of soil-borne pathogens following the treatment of soil with a specific chemical. For example, the incidence of tomato wilt caused by *Fusarium oxysporium* f. *lycopersici* was increased after the soil was treated with yellow oxide of mercury (20) and PCNB increased damping-off caused by a *Pythium* species (21). The enhancement of pathogenic activity could be caused by (1) a decline in the populations of organisms functioning to check the abundance or activity of the pathogen while the pathogen remains largely unharmed, or (2) a rapid reinfestation by the pathogen before its antagonists become sufficiently well-established.

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Recommended and suggested test protocols are presented in the style issued by the American Society for Testing and Materials.

3.1 PROCEDURE FOR THE EVALUATION OF A CHEMICAL'S EFFECT ON THE SOIL MICROBE POPULATIONS.

1. SCOPE

1.1 This procedure provides guidelines for determining the effect of a chemical on the populations of bacteria, fungi, and actinomycetes in soil. The number of colonies that appear on the growth medium provides an estimate of the number of viable propagules (spores, bacterial cells, and mycelial fragments) in the sample. All motions of the operator should be kept as standard as possible to minimize variability from human error.

2. SIGNIFICANCE

2.1 The equilibrium of the very delicate balance that exists between various types of microorganisms in the soil may be altered by chemicals in a variety of ways. First, there is the possibility that a chemical may have a toxic effect on a broad spectrum of microorganisms by inhibiting some essential metabolic activity, e.g., respiration. Second, the chemical may be selectively toxic to a certain group of microorganisms. Specific metabolic effects of this kind may alter population equilibria in an indirect manner by changing the competitive efficiency of one group or another. The third possibility is that the chemical may promote the growth of one or more types of soil organisms. The types favored could be either beneficial or harmful to soil fertility.

3.1 Standard Laboratory facilities for sterile culture.

- 3.1.1 Growth medium and glassware sterilizer
- 3.1.2 Glassware washing facilities
- 3.1.3 Incubators
- 3.1.4 Standard complement of glassware including: pipettes, petri dishes, flasks, balances
- 3.1.5 Medium preparation facilities
- 3.1.6 Sterile transfer facilities
- 3.1.7 Culture maintenance refrigerators

3.2 Materials for preparation of various media.

- 3.2.1 Agar
- 3.2.2 Mono-basic potassium phosphate
- 3.2.3 Di-basic potassium phosphate
- 3.2.4 Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)
- 3.2.5 Calcium chloride
- 3.2.6 Sodium chloride
- 3.2.7 Ferric chloride
- 3.2.8 Potassium nitrate
- 3.2.9 Calcium carbonate
- 3.2.10 Peptone
- 3.2.11 Dextrose
- 3.2.12 Rose bengal
- 3.2.13 Streptomycin
- 3.2.14 Asparagine
- 3.2.15 Mannitol

3.2.16 Glycerol

3.2.17 Sodium asparaginate

4. PROCEDURES

4.1 Dilution and plate count method.

4.1.1 A quantity of soil is serially diluted through water blanks and a suitable end dilution is plated on appropriate culture media. The number of colonies that appear provides an estimate of the number of viable propagules in the sample.

4.2 Growth medium

4.2.1 Thornton's standardized medium for bacterial culturing

4.2.2 Rose bengal agar for culturing fungi

4.2.3 Glycerol asparaginate medium for culturing actinomycetes

4.3 Soil preparation

4.3.1 Soil should be representative of the region(s) of concern.

4.3.2 Source of the soil is preferably from an agricultural field.

4.3.3 The characteristics of the soil should be defined as to soil type, composition of organic matter, sand, silt and clay, cation exchange capacity, pH, and field moisture holding capacity.

4.3.4 A composite bulk sample is obtained by collecting a number of random sub-samples, using a clean metal soil tube or soil auger, from the upper six inches of the field .

4.3.5 The composite soil mass is mixed thoroughly and then screened through a 2-mm sieve (U. S. Standard Sieve No. 10).

4.3.6 The soil should be processed immediately after collection and employed without delay .

4.3.7 Storage, if necessary, should be in a fresh state (not air-dried) at approximately 5°C and should not exceed 1 week.

4.4 Treatment of soil

4.4.1 Determine the average moisutre content prior to making aerial dilutions of the soil on three portions of 10 to 25 g dried for 24 hr at 100°C±5°.

4.4.2 All soil sample weights are subsequently based on oven-dry weight (o.d.).

4.4.3 Transfer at least 300 g of soil (o.d.) into a 600-ml beaker or other type of vessel suitable for mixing purposes .

4.4.4 Distribute evenly on the surface of the soil contained in separate mixing vessels a uniform but minimum amount of solution, made with the most appropriate solvent, to treat the soil with 0, 5, 25, 125, and 625 g of the test chemical per g of soil (o.d.), respectively.

4.4.5 Allow the solvent to evaporate from the soil, this may facilitated through the use of a gentle stream of filtered air taking precautions into account for volatile characteristics of the test chemical .

4.4.6 Mix the soil thoroughly with a heavy duty glass rod to insure that the chemical is distributed evenly throughout the soil sample. Use a standard technique that will yield mixtures of uniform distribution of the chemical and no great variability in organic solvent content .

4.4.7 Transfer equal quantities of the treated sample into each of three 250-ml Erlenmeyer flasks, properly labeled as to treatment and replication.

4.4.8 Remove approximately 10 g of the soil sample from each flask for moisture determination .

4.4.9 Transfer a weighed aliquot of each sample in the range of 1 g (o.d.) into a 99-ml sterile distilled water blank, to be used for propagule enumeration at 0-hr.

4.4.10 Add a sufficient amount of sterile distilled water to the soil in the Erlenmeyer flasks to adjust its moisture content to 75% of field moisture capacity

4.4.11 Close flask with Morton steel closures, plastic foam or cotton plugs.

4.4.12 Incubate soil samples at 28°C at high relative humidity

4.4.13 Agitate the prepared milk dilution bottles on a a mechanical shaker for 30 minutes.

4.4.14 From the original dilution (1:100) make additional serial dilutions of 1:1,000, 1:10,000, and 1:100,000 using a uniform shaking period of 30 sec. and settling period of 30 sec. between each subsequent dilution.

4.4.15 Aliquots of 0.1 ml of the desired end dilution is transferred to each of a minimum of 5 sterile petri dishes of the appropriate agar medium .

4.4.16 Inoculate the surface of the plates with a flamed glass rod while the plate is being rotated.

4.4.17 Incubate the plates at 28°C at high relative humidity.

4.4.18 At the end of 2, 4, and 7 days of incubation count the number of fungal, bacterial, and actinomycetes colonies, with the aid of a magnifier such as the Quebec colony counter, on the Thronton's, rose bengal, and glycerol-asparaginate agar media, respectively.

4.4.19 Repeat 4.4.8, 4.4.9, and 4.4.13 through 4.4.18 after intervals of 7, 14, 28 and 56 days of incubation.

5. DATA REPORTING

5.1 Express the number of progagules per gram of soil (o.d.) and analyze the results statistically for treatment and sampling times, factorily accounting for the variations due to replicate vs. treatments, plates x sampling times vs. treatments, and among plates .

6. PROCEDURAL MODIFICATION

6.1 The above procedures are suggested with the assumption that the worker who uses them is familiar with the fundamentals of microbial physiology and culturing of microorganisms. Aseptic techniques should be used wherever appropriate to avoid inadvertent contamination.

6.2 Variations of the basic procedures are conceivable, however, shortcuts to reduce the amount of glassware and number of steps may also reduce precision. Improved precision can be gained by employing a large sample, ca. 1,000 g but not to exceed a depth of 1 to 1 1/2 in., and using a soil aliquot for analysis of 25 g for the first dilution made with a total volume of 250 ml of 1% carboxymethyl cellulose or 0.2% agar. (7.4)

7. REFERENCES

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7.4 Curl, E. A. and Rodriguez-Kubana, R. *Research Methods in Wood Science.* Ed. R. E. Wilkinson. Creative Printers, Griffin, Georgia, pp. 161-194; 1972.

3.2 EVALUATION OF A CHEMICAL'S POTENTIAL TOXICITY TO THE MAJOR SOIL NITROGEN CYCLE BACTERIA

1. SCOPE

1.1 This suggested procedure provides guidelines for the evaluation of the potential toxicity of a chemical to the two major soil nitrification bacterial genera *Nitrosomonas* and *Nitrobacter*. This procedure is not intended to be a final authoritative and restrictive protocol but rather a guideline for studies from which further refinement and sophistication may be developed.

2. SIGNIFICANCE

2.1 The conversion of protein and other organic nitrogen compounds to nitrates for plant utilization , as well as the loss of nitrogen through nitrate leaching or denitrification, depends on this bacterial conversion. Substances which exhibit a high level of toxicity to *Nitrobacter* and *Nitrosomonas* represent a potential threat in many natural plant community soils as well as crop soils. Where materials exhibit significant toxicity at concentrations that would approach those occurring in soils through environmental release of the material, further evaluation is indicated. However, in certain agricultural soils nitrification inhibition is desirable and is done intentionally through various chemical compounds marketed for this purpose.

3. EQUIPMENT AND FACILITIES

3.1 General chemistry laboratory equipment and supplies.

3.1.1 Soil perfusion apparatus i.e. the Audus apparatus (7.2)

3.1.2 Sandy loam soil.

3.1.3 Appropriate reagents for nitrite and nitrate analysis in liquid solutions.

4. TEST PROCEDURE

4.1 Select source of sandy loam soil which is free from known chemical use or chemical contamination.

4.1.1 Sieve soil to remove coarse and fine particles. Collect aggregates of 2-4 mm size and air dry (in laboratory).

4.2 Soil bacterial enrichment.

4.2.1 Using an Audus apparatus, or other appropriate technique, continuously perfuse soil with 6.2×10^{-3} M ammonium sulfate solution (starting concentration) for 20-25 days.

4.2.2 Check nitrification through chemical analysis.

4.2.3 After 25 days, drain soil columns and wash with distilled water. Perfuse with fresh ammonium sulfate (starting concentration) until chemical analysis indicates a constant maximum rate of nitrification.

4.2.4 Wash soil samples with successive changes of distilled water and prepare for use.

4.3 Assessment of toxicant effects on nitrification process.

4.3.1 Prepare a series of perfusion columns each containing 50g of bacterially enriched soil (from the same enrichment procedure).

4.3.2 Replicate design treatments appropriately for statistical analysis of the data.

4.3.3 Perfuse columns at room temperature with 200 ml of 6.2×10^{-3} M $(NH_4)_2 SO_4$ containing concentrations of the test chemical ranging from .01 µg/ml to 1000 µg/ml in a $\log_{10}$ progression.

4.3.4 Various formulation procedures may be needed to keep the test substance in solution or in suspension. In these instances appropriate checks should be introduced to evaluate adjuvant effects.

4.3.5 Conduct perfusion for 10 days; withdrawing 1 ml samples daily for nitrate and nitrite analysis.

4.3.5.1 Nitrite detection may be performed by the Lees & Quastel method using the Illosvay reagent or other suitable technique (7.2).

4.3.5.2 Nitrate analysis may be done using the phenoldisulphonic acid method of Lees & Quatsel or other suitable technique (7.2).

4.3.6 In the absence of a toxicant, nitrite buildup is normally linear during the 10 day period.

5. PROCEDURAL MODIFICATION

5.1 The general procedures outlined here may be modified as needed to increase accuracy and effectiveness of the measurement technique. This general procedure is based on Debona and Audus (7.3).

6. DATA REPORTING

6.1 If the test substance will be introduced into soils (under manufacturing or use situations) in quantities approaching 0.01 the level that results in a 50 percent reduction in nitrification after 10 days of perfusion, then further detailed testing is suggested prior to environmental release.

7. REFERENCES

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7.3 Debona, A. C. and L. J. Audus. Studies on the effects of herbicides on soil nitrification. *Weeds Res*. 10: 250-263; 1970.

3.3 EVALUATION OF CHEMICAL PHYTOTOXICITY TO SOIL FUNGI OF IMPORTANCE IN CELLULOSE DECOMPOSITION

1. SCOPE

1.1 Guidelines are given to evaluate the potential toxicity of a compound to cellulose decomposing fungi in soil. It is not intended as a definitive or all-inclusive procedure but it will indicate the level of toxicity exhibited by a material under laboratory as well as simulated soil conditions. Testing may be conducted by persons competent in mycological/microbiological laboratory procedures in a general microbiological laboratory.

2. SIGNIFICANCE

2.1 Cellulose decomposition is an essential portion of the earth's carbon cycle. High levels of toxicity exhibited by a material in this procedure indicates the need for further testing in those instances where release of the material to soil through air, water, or direct placement is anticipated.

3. EQUIPMENT AND FACILITIES

3.1 Laboratory facilities for the sterile culture of fungi.

3.1.1 Growth media and glassware sterlizer.

3.1.2 Glassware washing facilities.

3.1.3 Incubators.

3.1.4 Normal complement of glassware including pipettes, petri dished, flasks, balances, etc.

3.1.5 Medium preparation facilities.

3.1.6 Sterile transfer facilities.

3.1.7 Culture maintenance refrigerators.

4. TEST ORGANISMS-SOURCE AND IDENTIFICATION

4.1 American Type Culture Collection

4.1.1 A.T.C. #9095 *Myrothecium verrucaria* Ditmar ex Fries maintenance medium, A.T.C. malt extract #325 .

4.1.2 A.T.C. #24687 *Trichoderma viride* Persoon ex Fries maintenance medium, A.T.C. potato dextrose agar #336 .

4.1.3 A.T.C. #6205 *Chaetomium globosum* Kunz ex Fries Maintenance medium, A.T.C. mineral salts agar #329 and filter paper.

4.2 Maintain test species under conditions that will insure stability and similarity of the isolate over time. This should prevent changes in response pattern to chemical exposure.

5. TEST PROCEDURE (8.1 and 8.2 with modifications)

5.1 Agar culture "poisoned food" technique.

5.1.1 Growth medium should be malt extract agar (ATC #325) amended with the test chemical and poured into petri dishes.

5.1.2 Dissolve or suspend test material in sterile water or appropriate sterile solvent prior to blending.

5.1.3 Pour plate technique should take into account test material solubility, solvent toxicity, or other problems associated with achieving a uniform mixture of test substance with the malt agar.

5.1.4 Agar should be partially cooled prior to toxicant addition to prevent change in test material. Pour and cool agar immediately after amendment.

5.1.5 Test material dosage should be calculated on the basis of chemical wt. to agar volume relationship i.e. μg/ml of agar. Initial dosages should be on a $\log_{10}$ progression beginning at 0.01 μg/ml and progressing to 1000 μg/ml.

5.1.6 Seed plates with uniform discs of fungal mycelium cut from equi-age (same radius) mycelium of colonies of the fungus grown in malt agar in petri dishes.

5.1.7 Replicate and design treatments appropriately for statistical analysis of the data.

5.1.8 Incubate "seeded" plates near the optimum temperature for vegetative growth of the fungi (24°C).

5.1.9 Record growth data by measuring the diameter of the fungal colonies when the growth leaves the original disc and prior to reaching the outer edge of the petri plate. Fungal growth is linear with time across agar. Radial growth may be calculated in the following manner:

$$\text{radial growth in mm/day} = \frac{\text{colony diameter day Y} - \text{colony diameter day X}}{2 \times \text{no. days growth } (Y - X)}$$

5.2 Soil amendment technique (8.12 with modification)

5.2.1 Fine sandy loam(pH 7.0-7.5)should be air dried, sieved through a 20 mesh screen and sterilized fro 45 min. at 15 psi in an autoclave.

5.2.2 The test material in a volatile solvent or in a finely divided state should be mixed with the dry sterile soil and then diluted with soil further so that a series of soil concentrations of 0.01 μg per ml air dry soil to 1000 μg per ml soil in a $\log_{10}$ progression can be established.

5.2.3 Place the soils containing the test dosages in sterile 25 ml shell vials (20 mm diam x 85 mm deep) to a depth of 25 mm.

5.2.4 Place A 10-mm disc from the rapidly growing margin of a fungal colony on malt extract agar on the soil surface. Cover the disc with 25 mm of soil containing the same concentration of chemical.

5.2.5 Add five (5) ml of sterile distilled water; plug the vials with cotton and incubate for 24 hours at 24°C.

5.2.6 Empty each vial onto a wire screen. Wash the disc with sterile distilled water and blot on sterile paper with towels. Place discs on malt extract agar to determine viability by visible growth from the disc after 48-72 hours.

5.2.7 Record data in terms of growth (+) or no growth (-). Estimate an ED_{50} for each test chemical.

5.2.8 Experimental design should allow for statistical analysis of the data.

6. MODIFICATION OF PROCEDURES

6.1 The preceding "poisoned food" and soil amendment procedures have been widely used by various researchers. Variations among the procedures are common. Modifications may be made by utilizing good experimental practice and design.

7. DATA INTERPRETATION

7.1 If the test substance is introducted into the soil in quantities equivalent to 0.01 ED_{50} for inactivation of fungal discs in soil, then further testing of the material is in order prior to intentional or unintentional soil introduction. This also applies to the "poisoned food" agar plate if there is complete growth inhibition at 0.01 µg/ml of agar (or less).

8. REFERENCES

8.1 Bateman, E. The effect of concentration on the toxicity of chemicals to living organisms *USDA Tech. Bull* 346 1-53; 1933.

8.2 Brancato, F. P. and N. S. Golding. The diameter of the mold colony as a reliable measure of growth *Mycologia* 45: 848-854; 1953.

8.3 Zentmeyer, G. A. A laboratory method for testing soil fungicides with Phylopthora cinnamomi as test organism. *Phytopathology* 45: 398-404; 1955.

3.4 ADDITIONAL LISTINGS TEST PROCEDURES FOR SOIL MICROFLORA

Soil forms the basis for terrestrial plant ecosystems. The nutrients released as a result of the microbiological interaction with inorganic and organic soil constituents are essential for terrestrial plant growth.

Substances toxic to soil microflora components including nitrification bacteria, cellulose decomposing fungi and soil algae may reach soils through water or air transport mechanisms. The research conducted in this area and the resultant publication of assay methods has not been directed towards pollution effects. Rather the work has revolved around pesticide effects on these processes or, in the case of soil fungi, the procedures have been developed to screen and evaluate potential chemical compounds toxic to fungi to be used as fungicides against soil-borne and air-borne plant pathogens. The procedures listed in most instances have not been used for evaluation of pollutant effects and thus should be modified and adapted to the purpose at hand, recognizing that the specific chemical and exposure route may modify the test procedure.

3.4.1 Nitrification

Nitrosomonas and *Nitrobacter* - major most significant bacteria in nitrogen cycle.

1. Effect on *Nitrosomonas* $NH_4^+ \; NO_2^-$
   Nitrobacter $NO_2^- \; NO_3^-$

Soil perfusion to achieve soil saturated with nitrifying organisms.

Debona, A. C. and L. J. Audus. Studies on the effects of herbicides in soil nitrification. *Weeds Res.* 10: 250-263; 1970.

2. Effect on *Nitrosomonas* NH_4+ NO_2^-

Use of specific *Nitrosomonas* inhibitor in comparison with non-treated checks and test substances.

Thorneburg, R. P. and J. A. Tweedy. A rapid procedure to evaluate the effect of pesticides on nitrification. *Weed Sci.* 21: 397-399; 1973.

3.4.2 Soil Algal Growth

1. Green algae *Chlamydomonas* and *Chlorella*.

Autotrophic and heterotrophic growth. Liquid culture.

Loeppky, Carol and B. G. Tweedy. Effects of selected herbicides upon growth of soil algae. *Weed Sci.* 17: 110-113; 1969.

2. Bluegreen algae *Cylindrosporium*

Green algae *Chlorella*, *Chlorococcum*.

Solid and liquid culture. Autotrophic growth.

Arvik, J. H., D. L. Wilson, and L. C. Darlington. Response of soil algae to picloram 2,4-D mixtures. *Weed Sci.* 19: 276-278; 1971.

Above procedures based on Jansen et al.

3. Green algae *Chlorella pyrenoidosa*

Chlorella pyrenoidosa--great deal of research knowledge as well as important soil alga.

Simple quick method. Semi-solid media. Probably applicable to bluegreen algae.

Thomas, V. M. Jr., L. J. Buckley, J. D. Sullivan, and Miyoshi Ikawa. Effect of herbicides on the growth of *Chlorella* and *Bacillus* using the paper disc method. *Weed Sci*. 21:449-451;1973.

Jansen, L. L., W. A. Gentner, and J. L. Hilson. A new method for evaluation of potential algicides and determination of algicidal properties of several substituted urea and *s*-triazine compounds. *Weeds* 6: 390-398; 1958.

3.4.3 Soil Fungi Growth

The procedures listed below are suitable for testing the reaction of a variety of fungi to various chemicals; several of which determine the volatile toxic action of the test substance. These were developed for fungitoxicity procedures against plant pathogens; hence, a modification of the test organisms to soil fungi important in saprophytic cellulose or organic matter decay or normal soil fungal constituents. The suggested test fungi should be representatives of the following genera with the species selection at the option of the investigator:

*1. *Myrothecium sp. (Myrothecium verrucariae)*
2. *Fusarium sp.*
3. *Aspergillus sp.*
*4. *Trichoderma sp. (Trichoderma viride)*

*5. *Chaetomium sp. (Chaetomium globosum)*

1. Simulates soil incorporation of pollutant. Measures diffusibility.

   Munnecke, D. E. A biological assay of non-volatile diffusible fungicides in soil. *Phytopathology* 48: 61-63; 1958.

2. Measures kill of fungi and complete eradication of organisms from soil.

   Corden, M. E. and R. A. Young. Evaluation of eradicant soil fungicides in the laboratory. *Phytopathology* 52: 503-509; 1962.

3. Can be used with above designated fungi as well as the *Phytophthora* plant pathogen. A very comprehensive procedure.

   Zentmeyer, G. A. A laboratory method for testing soil fungicides with *Phylopthora cinramoni* as test organism. *Phytopathology* 45: 398-404; 1955.

4. Requires diffusibility. Simple, easy to do.

   Thornberry, H. H. A paper-disk plate method for the quantitative evaluation of fungicides and bacteriacides. *Phytopathology* 40: 419-429; 1949.

**Myrothecium, Trichoderma, & Chaetomium*-important genera in cellulose decomposition in soil.

5. Measures fungal spore toxicity.

Chinn, S. H. F. and R. J. Ledingham. A laboratory method for testing the fungicidal effect of chemicals on fungal spores in soil. *Phytopathology* 52: 1041-1044; 1962.

6. Separates the fungistatic from fungicidal toxicants.

Neely, D. and E. B. Himelick. Simultaneous determination of fungistatic and fungicidal properties of chemicals. *Phytopathology* 56: 203-209; 1965.

7. These two procedures allow measurement of the toxicity of materials by volatile vapor phase action.

Richardson, L. T. and D. E. Munnecke. A bioassay for volatile toxicants from fungicides in soil. *Phytopathology* 54: 836-839; 1964.

Latham, A. J. and M. B. Linn. An evaluation of certain fungicides for volatility, toxicity and specificity using a double petri dish diffusion chamber. *Plant Dist. Reptr.* 49: 398-400; 1965.

3.5 PRELIMINARY EVALUATION OF TOXICITY TO TERRESTRIAL PLANTS THROUGH LABORATORY SEED GERMINATION AND SEEDLING GROWTH TESTING

1. SCOPE

1.1 These procedures will allow evaluation of toxicity of chemical configurations which are inhibitory to seed germination or seedling growth in the first three weeks after emergence. The procedures are not suitable for measurement of long term growth effects or for viewing maturational or reproductive effects.

2. SIGNIFICANCE

2.1 Many plant species are most sensitive to toxicants in the seed germination or seedling stage. Seeds or seedlings occupy a minimum of space and do not create the logistic problems of full scale field experiments. High levels of toxicities in seed or seedling tests strongly suggest the need for further greenhouse or field tests.

3. EQUIPMENT AND FACILITIES

3.1 Glassware and supplies associated with general horticultural and plant research laboratory.

3.2 Seed germinator or dark cabinet capable of maintaining a reasonably uniform temperature in the 21-27° C. range.

3.3 Plant growth facility-alternatives.

3.3.1 Plant growth chambers. Many types of controlled environment systems for raising plants are available commercially and their use in research is increasing. The systems vary considerably in size and capability, ranging from simple lighted cabinets to those providing rigorous control of several environmental parameters. Growth chambers are used primarily to (1) obtain uniform plant material, (2) permit selection of environmental conditions appropriate for a given plant species without regard to season, and (3) permit environmental manipulation as an experimental variable. If more than one type of growth chamber is available, the selection of the most appropriate one will depend on its intended use.

In general, all growth chambers contain mechanical, electrical, and perhaps electronic components and controls, all of which are subject to breakdown and require maintenance. The more sophisticated chambers generally provide greater flexibility of operation but often require more frequent or more complicated maintenance. Usually the simplest chamber that will meet the required environmental conditions is the best and often the least expensive. However, satisfactory control of a few major parameters will result in uniform and reproducible plant growth.

Temperature control is usually achieved through thermostatically controlled refrigeration and heating systems. It is useful to have two thermostats, one to control day temperature and the other for night temperature, with a timeclock to switch from one to the other. Switching need not coincide with the light-dark cycle. Temperature control of ± 2.0° C. is adequate for many purposes. Continuous temperature programming is provided in some growth chambers, but it is not needed for most experimental work. The degree of temperature control provided in an ordinary laboratory or workroom may be adequate for many purposes, and the on-off cycling of adequately ventilated banks of lights will provide some day-night temperature differential.

The lighting for growth chambers is commonly provided by a combination of fluorescent and incandescent illumination, banks of which commonly occupy the entire ceiling. The quality of the light from artificial lamps is not equal to sunlight. However, a satisfactory light quality may be achieved by using approximately 4 watts of cool-white fluorescent illumination per watt of incandescent illumination. The incandescent lamps need not be larger than 60 watts, although 100-watt lamps are sometimes used.

Control of day length is an essential part of environmental regulation. Timeclocks that can be set to turn the lights on or off at any 15-minute interval are satisfactory for most purposes. Regulation of light intensity is most commonly achieved by a combination of varying the distance between the plant bed and the light bank and by controlling the number of lamps lighted at any given time. Most growth chambers contain a mechanism for adjusting the plant bed height and several timeclocks, each controlling a part of the lights. Although illumination as low as 400 ft-c may be desirable for some purposes, most plants will grow satisfactorily in a white-walled chamber under a measured illumination of 1000 to 2500 ft-c.

Many growth chambers can provide illumination considerably in excess of 2500 ft-c; however, the literature reveals that 1600 to 2000 ft-c is usually sufficient for vigorous growth. Achieving maximum growth rates may require higher illumination.

The lamp bank and associated electrical ballasts generate a considerable amount of heat and this must be dissipated if temperature control is to be acheived. One of the better growth chambers has the ballasts in a compartment insulated from the plant growth space and a transparent barrier isolating the lamp bank.

Some chambers have a humidity sensing apparatus, which controls the operation of a refrigeration coil to trap unwanted water vapor, and a steam generator, wet pad, or aerosol generator to increase moisture in the air. In closed growth chambers without humidity control, precautions must be taken to insure adequate ventilation and thereby prevent the excessive moisture buildup that results from an enclosed space. Increased humidity may promote mildew development. If plants are grown under a light bank in a laboratory or workroom, humidity control is not practical and normal watering of the plants should be sufficient. A fresh-air change every 2 hours in the chamber is desirable to prevent excessive carbon dioxide buildup at night and a depletion during the day.

3.3.2 Laboratory tables or benches may be used with supplemental lighting by fluorescent plant growth tubes to 1600 ft-c on time clock control, and room temperature range from 21-27° C. These may be used in place of growth chambers.

3.4 Balances, scales, rules, and other plant measurement equipment.

4. TEST PROCEDURES

4.1 Test plant species.

Monocotyledons

Oats--*Avena sativa* L. 'Clintford'

Ryegrass--*Lolium perenne* L. 'Manhattan'

Corn--*Zea mays* L. 'Butter and sugar'

Dicotyledons

Cucumber--*Cucumis sativus* L. 'Marketer'

Bean--*Phaseolus vulgaris* L. 'Pinto'

Tomato--*Lycopersicon esculentum* Mill. 'Rutgers'

4.1.1 All six species should be tested with all procedures.

4.2 Seed germination.

4.2.1 Test materials should be prepared as solutions, suspensions, or emulsions in distilled water in concentrations ranging from 0.01 μg/ml to 1000 μg/ml in $\log_{10}$ progression including the control.

4.2.2 Pour 100 ml of the solution in a 250-ml Erlenmeyer flask containing 50 seeds. Place on a shaker for 24 hours at room temperature.

4.2.3 Remove seeds and wash in distilled water over a screen or Buchner funnel.

4.2.4 Place the washed seed on 3 layers of clean moist filter paper in a large petri dish (approx. 200 mm x 20 mm) and maintain in the dark in an incubator or germinator at 21-27° C. for 10 days. Determine the percent germination at the end of 5 and 10 days. A seed with any degree of sprouting is considered germinated.

4.2.5 The treatments should be replicated and the experimental design suitable for statistical analysis of data obtained. Appropriate controls and blanks should be included where emulsifiers or solvents were used to obtain solution or suspension of the material.

4.2.6 For each substance and test species an ED_{50} should be estimated. In this case the ED_{50} is that dosage in µg/ml solution which results in inhibition of 50 percent of the germinable (viable) seeds in the test population when compared with the germination percentage following distilled water treatment.

4.3 Seedling growth effects.

4.3.1 This method consists of transplanting seedlings into a vermiculite medium proviously amended with a test substance and then measuring growth effects after a 3-week period or transplanted seedling foliage may be sprayed with the test substance in aqueous solution and growth effects measured after a 3-week period.

4.3.2 Wet two paper towels and place on waxed paper. Fold back 5 cm. of the upper paper and place seed on the second paper along the fold. Fold back upper paper over seed. Loosely roll absorbent paper towels inside the waxed paper. Place in a beaker with 5 cm. of water and incubate 4-5 days at the desired temperature, in the dark. Remove seedlings from the incubator and expose to room light (100-200 ft-c) for a day. Transplant hardened seedlings and place in an environment with required illumination.

Seed germination by the "paper roll" method may require more time and care than direct seeding in flats. However, distinct advantages make this method worthwhile. Seedlings germinated by this method may be selected for uniformity of root and shoot development and may be transferred to vermiculite growth medium with minimum root damage and without particulate matter adhering to them.

4.3.3 Culture techniques.

4.3.3.1 Containers for the brief 3 week period utilized this evaluation should be disposable. Plants may

Magnesium sulfate.	$MgSO_4 \cdot 7H_2O$	246.5

Iron solution- The iron chelates of ethylenediaminetetraacetic acid (EDTA) are commercially available, often as Versene or Sequestrene. Dilute the commercial product to obtain a 5 percent w/v solution. Dilute 200 ml of this solution in distilled water and make to 1 liter. This stock solution contains 10,000 ppm of iron chelate. One ml of stock solution will give an iron concentration of 1 ppm when diluted to 1 liter.

Micronutrients- The microelements or tract elements are dissolved together in 1 liter of deionized water to make a stock solution. The microelements should be added to the water in the order listed and each dissolved before the next is added to avoid precipitation.

Chemical	Formula	Grams per liter
Boric acid.	H_3BO_3	2.50
Zinc chloride	$ZnCl_2$	0.50
Cuprous chloride.	$CuCl_2 \cdot H_2O$	0.05
Molybdenum oxide.	MoO_3	0.05
Manganese chloride.	$MnCl_2 \cdot 4H_2O$	0.50

Preparation of nutrient solution from stock solutions-The following volumes of the six stock solutions are then individually added to about 500 ml of deionized water with stirring. After all stock solutions have been added, the nutrient solution is made to 1 liter with deionized water to obtain full-strength solution. The solution is usually diluted to one-third, one-half, or three-fourths strength.

Chemical	Formula	Milliliters
Potassium dihydrogen phosphate	KH_2PO_4	1
Potassium nitrate	KNO_3	5
Calcium nitrate	$Ca(NO_3)_2$	5
Magnesium sulfate	$MgSO_4$	2
Iron		1
Micronutrients		1

4.4 Experimental design-The test procedures should be planned in such a manner that replications, pot arrangements, experimental and control organisms are suitable for appropriate statistical analysis. Special precautions in the design should allow measurement of the independent effects of chemical adjuvants, i. e. solvents, emulsifiers, etc. that are used in making the test substance suitable for soil amendment, spraying, or seed treatment.

5. PROCEDURAL MODIFICATIONS

5.1 The general procedures outlined here may be modified as needed to increase accuracy and effectiveness of the evaluation technique. The general plant cultural procedures recommended are taken from the report cited in reference No. 8.1

6. DATA REPORTING

6.1 Seed germination

6.1.1 Estimation of the ED_{50} for seed germination inhibition should be made for 5 and 10 days.

6.2 Seedling growth — soil treatment and foliar spray

Estimation of the LD_{50} dosage which results in the complete death of 50 percent of the test plants through root or foliage exposure should be made for each exposure method. In addition detailed records should be kept of plant heights, dry and fresh weights of top growth, and morphologic growth changes at the end of the 3-week period.

7. DATA INTERPRETATION

7.1 The germination and plant growth data should be reviewed against the anticipated dosages and concentrations that are likely to occur through environmental release of the test substance. If the environmental accumulation will be 0.01 times that concentration resulting in significant seed germination or seedling growth effects in the test procedures, then further greenhouse and field testing is suggested.

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3.6 GREENHOUSE EVALUATION OF TOXICITY TO TERRESTRIAL PLANTS THROUGH FOLIAR SPRAY AND SOIL AMENDMENT TESTING

1. SCOPE

1.1 These procedures represent a continuing expansion of the initial growth chamber laboratory bench seedling procedures. Greenhouse plant cultures can be adjusted with appropriate container sizes and plant species to examine the effects of a test substance on the complete growth cycle of a plant from seedling to mature plant and may in some circumstances include flowering and fruit production. In addition, interactions between potentially antagonistic or synergistic toxic substances may be examined.

2. SIGNIFICANCE

2.1 In some instances greenhouse testing allows interpretation of results as being representative of anticipated field results. The cost of greenhouse testing may be less than growth chambers. However, the greenhouse environment differs from the outdoors in light quality and temperatures. Therefore, greenhouse testing should not be considered a substitute for in situ field testing under actual toxicant release conditions. However, a long term greenhouse test provides a greater in depth evaluation of the toxicity hazard than a short term laboratory bench or growth chamber tests.

3. EQUIPMENT

3.1 Greenhouse with temperature and light control, headhouse facilities for potting, transplanting, etc.

3.1.1 Greenhouses

3.2 Growth media blending and mixing equipment

3.3 Pot or flat spraying equipment for uniform chemical applications.

3.4 Scales, balances, and plant measurement supplies.

3.5 Soil nutrient supplies.

4. TEST PROCEDURES-PLANT CULTURE AND PRODUCTION (11.1)

4.1 Greenhouse selection and maintenance. Many types of greenhouses are available commercially, and vary considerably in size, shape and construction materials. The covering may be glass or translucent plastic. Glass is more transparent, but it is susceptible to breakage and increases heat retention. Regardless of the type of greenhouse available, certain features are necessary to insure its usefulness for cultivating plants throughout the year. Provisions must be made for heating and supplemental lighting during winter. A reduction in temperature can be provided by a combination of shading, cooling, and ventilation.

Shading of greenhouses in the summer is essential to prevent excessive heat buildup. This may be accomplished either by painting the houses with a lime-linseed oil shading compound or by using a shade screen. The shading compound will weather during the summer, often providing inadequate shading during the last part of the season. Eventually it will etch the glass and reduce the transparency of the greenhouse during the winter.

Temperature may be controlled by using ridge or side vents. These are often used in conjunction with forced-air evaporative coolers, which bring air into the greenhouse. A high degree of summer temperature control can be achieved if both the evaporative coolers and motorized vents are thermostatically activated. The covering of vent openings with a fine screen will exclude birds and most insects, thereby reducing plant damage and the frequency of fumigation. Without cooling, the temperature in a greenhouse during the summer may range between 20-45° C.

Supplementary light controlled by timeclocks is best supplied by fluorescent lamps without reflectors. These provide minimum shading of plant benches when the sun is shining and can be left in place permanently. Four 244-cm fluorescent lamps over each 122 × 244 cm table will supply from 300 to 600 foot-candles (ft-c) of illumination, which controls day length and is sufficient to maintain many species above the compensation point during the winter. These lamps have an average life of approximately 7500 hours and can be used in the greenhouse for at least two winters.

With an overcast sky during the day, the natural illumination in the greenhouse may be 900 to 1100 ft-c, and on a bright day it may reach 5000 to 7000 ft-c.

The ideal situation is to cultivate each species under its specific optimum conditions. Because of limitations in cost, space, and time, it is often necessary to grow several species in the same greenhouse section or growth chamber. Therefore, compromises may have to be planned. When two species are grown together, neither will respond optimally to compromise of growth conditions. Every attempt should be made to grow plants together that have similar growth requirements.

Under varying environments the water and nutritional requirements of plants will differ. During the summer it may be necessary to water the plants twice daily, whereas once a day may be satisfactory in the winter. The same is true when nutrient solution is applied two or three times a week, alternating with water only. If the growth rate of the plants increases, additional nutrients are necessary. As plants approach maturity and growth rate decreases, their nutrient requirements are reduced. For certain species of plants, the flowering stage may be delayed or hastened according to the photoperiod they receive.

4.2 Cultural conditions. Illumination and quality of light, photoperiod, temperature, relative humidity, and nutrition all interact to affect plant growth. Less than optimum conditions can result in poor plant growth.

4.2.1 Light. The illumination, light quality, and photoperiod requirements vary among species. A foot-candle meter is often used to measure illumination. Although foot-candle meters can be obtained, photographic light meters are also practical for light measurement. Photographic light-meter readings can be converted into foot-candles by the following formula:

$$B = \frac{20\ (f)^2}{TS}$$

Where

B = illumination in foot-candles

f = aperture in f stop

T = shutter speed in seconds

S = film speed in ASA units

To measure illumination with a photographic light meter, reflected rather than incident light must be measured.

To do this, place a large sheet of white paper on the surface to be measured, set an appropriate ASA film speed on the meter, and read the shutter speed required for proper exposure at a given f stop.

Ft-c values for meter settings may be obtained by solving the equation. The results will be approximate depending on the accuracy of the meter and the cone of light it accepts. For example, false low readings may result if the meter accepts light from an area greater than that of the white paper at which it is directed. Nevertheless the readings can suffice to determine whether illumination is adequate for good plant growth. About 1200 ft-c of light in a growth chamber is satisfactory for many plants such as cabbage, carrots, peas, and tobacco, whereas other plants such as corn, cotton, rice and sorghum grow better when supplied with 1600 to 1800 ft-c.

Different plant species vary in their photoperiod requirements. For example, barley requires a photoperiod greater than 12 hours for good flowering, whereas soybeans can mature under a 12-hour day.

During the winter a 12-hour photoperiod is generally used as a compromise day length under which reasonable vegetative growth can be maintained. The short natural photoperiod in the winter is supplemented with fluorescent light.

4.2.2 Temperature. During the summer it is often difficult to maintain sufficiently cool temperatures in the greenhouse for certain species of plants. For example, head lettuce and peas will often grow poorly in midsummer. It is not recommended to grow these species until conditions are more favorable.

4.2.3 Relative humidity. An average of 50-percent relative humidity is satisfactory for many species. A relative humidity of 100 percent is often needed to germinate very small seeds such as those of tobacco, bluegrass or root sugarcane stem sections. The required humidity can be obtained by covering the plant containers with plastic bags.

4.2.4 Growth media. Vermiculite of a medium fine texture is readily obtained from local dealers. Local tapwater may be too contaminated, saline, or alkaline for use in nutrient culture. It may be deionized by passage through a commercial mixed-bed deionizer.

4.2.5 Nutrition. Environmental conditions affect the amount and rate of nutrient uptake by individual plants. If many different plants are to be grown, considerable variation in nutrient requirements can be expected. By careful observation, early deficiency symptoms can be diagnosed and readily cured. If fairly exact nutrient requirements are not known for a given species, it is best to use a standard solution, taking care not to overfertilize, for it is easier to correct a nutrient deficiency than an excess. Many plants seem to tolerate wide variations in nutrient supply. It is desirable to supply nutrients at the optimum level.

4.3 Seed germination. A temperature-controlled incubator may be used for seed germination. Most seeds are germinated in the dark, although some require light. If a dark incubator is not available, any dark cabinet in which the temperature remains fairly constant (21-27° C) can be used. Reasonably uniform and reproducible seed germination will be obtained.

4.3.1 Method 1. Fill a small flat, 30 × 20 × 10 cm, with 5 cm of vermiculite. Place a seed on the surface and cover with 1.3 cm of vermiculite for alfalfa seeds and 2.5 cm for pea seeds. Very small seeds, i.e.,tobacco, are mixed with sand to increase the volume for better distribution. These seeds are not covered after seeding. The vermiculite is wetted from the bottom. An enamel tray is placed under the flat to maintain the moisture level. The flat with tray is placed in an incubator at the desired temperature for the required length of time. If the seedlings are kept in flat for an extended period of time, a dilute nutrient solution can be used in place of water. Metal flats should be of stainless steel, or if galvanized they should be coated with an asphaltic material to prevent toxic levels of zinc from leaching into the germinating medium.

This method is generally the easiest and most successful for many types of seeds. The advantages of using vermiculite for germinating seed are as follows: (1) adequate moisture-holding capacity and aeration, (2) lack of toxic materials and nutrients, (3) root development encouraged (4) light weight and easy to handle. Although peat may be mixed with vermiculite for some purposes, its high absorptivity may interfere with subsequent chemical treatments applied to the roots.

4.3.2 Method 2. Wet two paper towels and place on waxed paper. Fold back 5 cm. of the upper paper and place seed on the second paper along the fold. Fold back upper paper over seed. Loosely roll absorbent paper towels inside the waxed paper. Place in a beaker with 5 cm of water and incubate 4-5 days at the desired temperature, in the dark. Remove seedlings from the incubator and expose to room light (100-200 ft-c) for a day. Transplant hardened seedlings and place in an environment with required illumination.

Seed germination by the "paper roll" method may require more time and care than direct seeding in flats. However, distinct advantages make the method worthwhile. Seedlings germinated by the paper roll may be selected for uniformity of root and shoot development and may be transferred to nutrient or other solutions with minimum root damage and without particulate matter adhering to them.

4.4 Culture techniques

4.4.1 Containers. The plant container must be of sufficient size to permit adequate root growth. For example, a pea plant can be grown to maturity in a 10-cm plastic pot filled with vermiculite or in a 500 ml jar filled with nutrient solution. A corn plant grown in a 10-cm pot or 500-ml jar becomes rootbound after 5 to 6 weeks of growth and abnormalities will occur. Corn plants require an 18 cm pot or 2 liter jar to grow to maturity.

For this reason various types and sizes of containers are used, such as jars, stainless-steel troughs with lids, and plastic pots with saucers. Milk cartons can also be used as disposable containers. They will often last for 6 to 8 weeks. Glass jars are covered with a coat of black paint followed by a coat of aluminum paint. The black paint excludes light, inhibits growth of algae in the nutrient solution, and prevents abnormal root pigmentation. Since the aluminum paint reflects sunlight, the jars remain cool.

When many plants are to be grown together in the same container in nutrient solution, use a stainless-steel trough, 66 × 16 × 10 cm, with a stainless-steel lid. The plants are placed in holes in the lid, which serves as a support and keeps the light out. Soft plastic collars may be used for additional stem support. The size of the holes should be adequate to prevent stem girdling. Alternatively the flat steel trough lids may be replaced by wood or plastic frames with nylon screen bottoms. These frames are supported within the troughs, above the bottom. The screen is filled with vermiculite. The plants can be grown directly from seed or transplanted. Nutrient solution is added to wet the vermiculite. The plant roots grow through the screen and into the nutrient solution. Many plants grow well in this manner. The screen gives good root support, and most of the roots can be harvested.

Plexiglass framing is preferred to wood to avoid leaching of chemicals from the frame. If the experiment requires root treatment by the addition of a chemical to the nutrient solution, a wooden frame may become contaminated and should be discarded.

If plants are grown in jars, then jar lids, tinfoil, waxed cork, Masonite or other materials can be used for plant support. Many people have found paper cups most satisfactory for support. They can be used in various ways depending on the species of plant to be grown. For example, when pea plants are grown, two cups are glued together on the bottom. The lower cup slips over the outside of the jar; the upper cup is used for plant support. One small hole is punched through the bottom of each cup for the seedling and another small hole through the lower cup for the aeration tube.

The rationale for this technique is: (1) the cups are chemically unreactive, (2) they may be discarded after use, (3) sharp cutting edges are eliminated, (4) girdling can be prevented by gradually enlarging the hole as the plant stem increases in diameter, (5) cups can be easily lifted so that nutrient solution can be added, (6) plants can be easily transferred to other jars, (7) there is more stem support for certain plants than with many other types of lids.

4.4.2 Aeration. When plants are grown directly in a liquid culture, air must be supplied to the nutrient solution. A small electric pump ,i.e., a fish tank aerator, can force air through rubber or plastic tubing to the jar or troughs. Glass capillary tubing is inserted in the solution. A well-regulated uniform flow rate can be obtained for several aerators on the same line by inserting a 2.5-cm length of 0.25-mm bore capillary tubing into the air line for each aerator tube. Aerating can be done continuously or at regular intervals, (two hours on, two hours off) by using a timeclock.

If compressed air is available, this may be more economical than using many small pumps, which require frequent maintenance. Oil vapors from a rotary-type compressor must be trapped out with a charcoal filter before the air reaches the plants.

4.4.3 Subirrigation. Plants grown in vermiculite-filled pots or flats should receive water and nutrient solution by subirrigation. Saucers or shallow pans are used under the pots and enamel trays under the flats. The amount of water or nutrient solution added is dependent on the plant and growing conditions. It is not as easy to overwater plants grown in vermiculite as it is in soil. Plants that require well-aerated media can become waterlogged if the vermiculite is watered excessively. Care must be taken to avoid this. When in doubt, consult those who have grown these plants, or refer to literature on the specific plant.

Some salt accumulation may occur on the surface of the vermiculite after a period of time, and this should be leached out once a month. This is done by applying excessive water at the top and letting it drain through the container. Subirrigation may then be resumed on the usual schedule. Salts are leached away very rapidly from vermiculite by this method. The pots and flats are watered from the top only at seeding time. When very small seeds are placed in flats and left uncovered, subirrigation is used. If they are watered from the top, a fine spray must be used to prevent the seed from being carried too deep into the vermiculite to emerge.

The following methods can be used to apply nutrients to plants: (1) mix the nutrients directly into the watering system (2) add nutrient solution two or three times per week and water at other times (3) supply a nutrient solution every day without mixing it in the watering system; the concentration can then be varied as needed. When using method 2, tapwater can often be substituted for distilled or deionized water if the plants are not going to be used for a critical experiment in which nutrition should be carefully controlled. The use of tapwater several times per week reduces the volume of deionized water required. However, it must be employed with caution, since some domestic water supplies may contain levels of salts that will damage plants. Soft water is not necessarily low in salt content since the softening process exchanges highly soluble salts for less soluble ones.

The acidity or alkalinity of the water used may also affect plant growth adversely. Some injurious pH effects result from changes in the availability of nutrient salts. For example, excessive watering of corn with alkaline (pH9) tapwater can cause the leaf margins to become chlorotic and torn. These symptoms are reminiscent of certain nutrient deficiencies. The addition of dilute acids, bases, or buffering salts to the tapwater or nutrient solution can help overcome these difficulties. Care must be taken, however, to insure that neither the nutrient solution nor the tap-water used between additions contains too much salt. It is necessary to experiment in order to determine the optimum amounts of nutrient for individual species.

4.4.4 Nutrient solution. The following modified formula for Hoagland and Arnon should be used. Preparation is as follows:

Major elements.--Individual 1 M stock solutions of each major element are made with deionized water.

Chemical	Formula	Grams per liter
Potassium dihydrogen phosphate.	KH_2PO_4	131.1
Potassium nitrate.	KNO_3	101.1
Calcium nitrate.	$Ca\ (NO_3)_2 \cdot 4H_2O$	236.2
Magnesium sulfate.	$MgSO_4 \cdot 7H_2O$	246.5

Iron solution. The iron chelates of ethylenediaminetetraacetic acid (EDTA) are commercially available, often as Versene or Sequestrene. Dilute the commercial product to obtain a 5 percent w/v solution. Dilute 200 ml of this solution in distilled water and make to 1 liter. This stock solution contains 10,000 ppm of iron chelate. One ml of stock solution will give an iron concentration of 1 ppm, when diluted to 1 liter

Micronutrients. The microelements or trace elements are dissolved together in 1 liter of deionized water to make a stock solution. The microelements should be added to the water in the order listed and each dissolved before the next is added. This prevents precipitates from forming.

Chemical	Formula	Grams per liter
Boric acid.	H_3BO_3	2.50
Zinc chloride	$ZnCl_2$	0.50
Cuprous chloride.	$CuCl_2 \cdot H_2O$	0.05
Molybdenum oxide.	MoO_3	0.05
Manganese chloride.	$MnCl_2 \cdot 4H_2O$	0.50

Preparation of nutrient solution from stock solutions. The following volumes of the six stock solutions are then individually added to about 500 ml of deionized water with stirring. After all stock solutions have been added, the nutrient solution is made to 1 liter with deionized water to obtain a full-strength solution. This solution is usually diluted to one-third, one-half, or three-fourths strength.

Chemical	Formula	Milliliters
Potassium dihydrogen phosphate.	KH_2PO_4	1
Potassium nitrate.	KNO_3	5

Calcium nitrate	$Ca(NO_3)_2$	5
Magnesium sulfate	$MgSO_4$	2
Iron		1
Micronutrients		1

5. TEST PROCEDURES.--CHEMICAL EXPOSURE METHODS

Test substances may be applied to plants either through foliar spray or root contact through growth media amendment. A liquid or vermiculite culture is suitable for the foliar spray technique. A vermiculite culture is most appropriate for growth media amendment. Both foliar spray and soil amendment exposure methods should be tested.

5.1 Foliar spray techniques may be performed by one of two techniques. The first is through foliar contact. The test solution is sprayed with an atomizer to the point of run-off. Tip the pot or cover the medium surface to avoid chemical contact. The alternative method is to spray pots or flats from overhead with both foliage and vermiculite surface receiving chemical contact with a known surface area dosage. In this manner, a known surface area dosage of chemical contacts both the foliage and vermiculite surface.

5.2 Growth media amendment should be done prior to transplanting. The test substance is mixed with water to form a solution, suspension, or emulsion. This is combined with the vermiculite using enough diluent to achieve thorough blending. Dosages should be calculated on a weight/volume relationship with the test dosages ranging from 0.01 μg/ml growth media (vermiculite) to 1000 μg/ml on a $\log_{10}$ progression. Uniform amounts of the amended media should be placed in each container and the seedlings transplanted. The seedling and container should be subirrigated with an individual pan or petri dish "half" beneath each container. Nutrient concentrations should be weak to minimize the need for periodic leaching from above to reduce salt concentrations, which may leach out the test substance.

6. TEST PLANT SPECIES

6.1 Monocotyledons

Oats- *Avena sativa* L. 'Clintford'
Ryegrass- *Lolium perenne* L. 'Manhattan'
Corn- *Zea mays* L. 'Butter and sugar'

6.2 Dicotyledons

Cucumber- *Cucumis sativus* L. 'Marketer'
Bean- *Phaseolus vulgaris* L. 'Pinto'
Tomato- *Lycopersicon esculentum* Mill. 'Rutgers'

6.3 All six species should be tested with all procedures.

6.4 In addition to these six basic species other additional species and cultivars may be used as appropriate.

7. DURATION OF TESTING

7.1 The basic test period varies depending on the needs of the evaluation. Foliar tests may involve seedling sprays, repeated sprays, or mature plant sprays, depending on the test objective. The duration of the experiment may proceed through plant maturity, including flowering and reproduction.

8. PROCEDURAL MODIFICATION

8.1 The general procedures outlined here may be modified as needed to increase accuracy and effectiveness of the evaluation. For certain purposes, natural soils may be used instead of vermiculite. (11.3)

9. DATA REPORTING

9.1 Potential effects of test substances are listed below. The effects denoted by an asterisk should be determined in all instances.

9.1.1 Visual changes in morphology.

9.1.1.1 Above ground.

a. foliage*- leaves, petioles, foliar arrangements size, shape, distortion, color.

b. support structure*- stems, limbs, stolons, tillers size, shape, distortion, color

c. **reproductive organs*- flowers, fruits, seeds size, shape, distortion, color.**

9.1.1.2 Below ground.

a. **rhizomes, tubers, corms, bulbs--size, shape, distortion, color.**

b. roots*- size, shape, distortion, color.

9.1.2 Changes not visually detectable.

9.1.2.1 Anatomical--tissue and cellular arrangement.

9.1.2.2 Physiological.

a. Chemical constituents.

1. nutrients, vitamins, etc.

2. metallic and non-metallic elements.

3. other organic and inorganic constituents.

b. Crop plant yields or other subtle changes in morphology which are not visually detectable but only discernible through quantitative measurements*, i. e. yield, height*, weight*, etc.

c. Changes in reproduction.

1. mutations
2. progeny abnormalities in F_1 populations.

10. DATA INTERPRETATION

10.1 Interpretation in comprehensive greenhouse testing will be complex. At this stage in testing a great deal may be learned about the potential physiologic effects of a substance. The major problem is to determine if such greenhouse-demonstrated effects are likely to occur under natural conditions.

10.2 If a given substance does exert profound physiologic effects at low levels and if significant amounts reach the environment in soils and waters or will be deposited directly on plants, then the rule of 0.01 (11.5) should apply. That is, if the anticipated environmental concentration and exposure route will reach 0.01 the dosage required to exert physiologic effects in the greenhouse tests, then field testing should be initiated. This includes in situ testing with species occurring in the anticipated release areas as well as the six standard species.

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11.4 Wilcox, M. A. sprayer for application of small amounts of herbicides to flats. *Weed Sci.* 16: 263-264; 1968.

11.5 Lehman, A. J., FDA, *Federal Register* p. 1493. March 11, 1955.

3.7 EVALUATION OF PLANT TOXICITY THROUGH FIELD TESTING

1. SCOPE

1.1 These guidelines will allow development of field plot or field-scale testing of potential toxicants. From these procedures the scale may vary from small/centare size individual plots to hectare size whole field plots. Testing may be conducted on the site of the actual anticipated release or simulated areas. Although the basic six species used for growth chamber and greenhouse testing are suggested for inclusion, additional species may also be added as appropriate.

2. SIGNIFICANCE

2.1 The underlying premise for field evaluation is that such experimentation will be representative of the effects of toxic substances under actual commercial manufacture and release situations. How well such experiments simulate reality depends on the soundness of the experimental design. Properly conceived experiments will predict accurately the actual effects of toxicants and may be used as the final criteria in manufacturing and environmental release situations.

3. EQUIPMENT AND FACILITIES

3.1 Suitable experimental land areas, i.e., field plot sites, must be free from chemical treatment history. These sites should be level and uniform in all directions.

3.2 Soil tillage and planting equipment should provide uniform results with minimal variation. The capability of the equipment depends on the extensiveness of the plot.

3.3 Test substance application equipment.

3.3.1 Granule applicators and incorporation blenders.

3.3.2 Even-dosage plot sprayers.-gasoline, electric, compressed gas and hand-powered. (7.3)

3.3.3 Logarithmic sprayers.-electric, compressed-gas and hand-powered. (7.3)

3.4 Surveying, measuring and weighing equipment.

3.4.1 Tapes, transits, stakes, rulers, etc.

3.4.2 Scales, balances, etc.

4. TEST PROCEDURES

4.1 Test plant species

Monocotyledons

Oats--*Avena sativa* 'Clintford'

Ryegrass--*Lolium perenne* 'Manhattan'

Corn--*Zea mays* L. 'Butter and sugar'

Dicotyledons

Cucumber--*Cucumis sativus* L.'Marketer'

Bean--*Phaseolus vulgaris* L. 'Pinto'

Tomato--*Lycopersicon esculentum* Mill. 'Rutgers'

4.1.1 All six species should be tested with all procedures.

4.1.2 In addition to the six species above, other species should be included, depending on the vegetation and crops of the exposure area(s).

4.2 Field plot experimental design

4.2.1 Certain principles involved in the use of field plots must be considered. Variability in the test plot area soil is an outstanding problem. Soil is universally heterogenous. Consequently, any two plots taken at random in a field and sown with the same kind of seed will almost always fail to give the same plant growth. Error is reduced if conspicuous variations in soil are avoided which makes the choice of land a major consideration.

Placing two plots close together ordinarily will make them more alike than if they are some distance apart. Hence, there is a positive correlation between the response of plots placed close together. For example, in comparing two soil toxicant treatments, it follows from the nature of soil variability that they should be near one another. Similarly, for more that two treatments it is essential that all the plots be placed in a compact group.

4.2.2 Plot shape, plot size- The shape of long narrow plots makes it possible to bring them closer together than square plots. Severe practical restrictions on the narrowness of plots are certain considerations, such as border effects, diffusion of treatments, and the mechanics of seeding, cultivation, and evaluation. The ideal plot provides a proper balance between these considerations.

The size of the plot is more difficult to decide. For example, small seeded cereal grain plots are commonly 3-4 rows wide and 5 m long for preliminary tests and in 1/100 hectare plots for final tests. To avoid margin effects only the center rows are harvested.

4.2.3 Replications.- Replications are essential because of the variables present in field experiments. The usual plan is to have a single plot of each treatment in a compact group or block and to replicate the blocks as often as necessary to secure the desired accuracy. This gives some degree of control over the error of the experiment, and furnishes the mechanism for determining the experimental error, which cannot be obtained otherwise.

4.2.4 Plot plan (7.1.1). Many plot plans are possible; but two are commonly used: the randomized block and the Latin square.

4.2.4.1 Randomized Block. This method consists of replicate blocks, containing a number of rows equal to the number of treatments. Treatments are assigned at random. The shapes of the entire planting of the replication blocks, and of the individual treatment plots within the replication blocks are important for error control. The smallest units, square treatment plots, are less likely to respond similarly than long narrow plots placed side by side. In many situations a long narrow arrangement (within limits) may be preferable. Similarly, the differences that exist in the soil selected, should be as great as possible between replication blocks. Thus, the shape of these blocks should be as nearly square as possible. The most compact and suitable arrangement approaches a square. However, local conditions may indicate a modification.

4.2.4.2 Latin Square. This plan is accurate when the number of treatments is eight or less. The field is rectangular and divided into an equal number of rows and columns. Each treatment will occur once in each row and column. The advantage of the Latin Square is that it controls variability of soil, etc., in two directions across the field. This is particularly valuable when the direction of the important fertility trends cannot be predicted. Although plots may be rectangular, the efficiency of the Latin Square to the randomized block is less obvious with an increase in length and width.

4.2.5 Miscellaneous problems. The effects of competition between a plant and its neighbor are sometimes manifested at the edge of experimental plots unless precautions are taken to prevent them. It is desirable to arrange for extra plants on the ends and sides of an experimental plot to equalize the competition between the plants under examination. This is considered in cases where groups of plants in the trials are either killed or conspicuously stunted by the test substance treatment.

Also, the rate of seeding must be taken into consideration. Ordinarily, the optimum rate of slightly above is employed. This factor is usually not of consequence except where the size of seed in different varieties varies greatly and the competition between plants, and between rows, influences the result.

4.3 Test plant establishment.

4.3.1 Test plants may be established through seeding by hand or with mechanical seeders.

4.3.2 Test plants may be established through hand transplanting or through mechanical transplanters.

4.3.3 In the case where the test substance is employed as a soil amendment, seeding or transplanting may be done after the soil amendment has taken place.

4.4 Test substance application techniques.- Test substances may be applied to plants and soils in the field in the following ways:

4.4.1 Spraying or spreading the substance on the soil surface then "rototilling" or discing into soil profile, followed by planting.

4.4.2 Spraying of spreading on soil surface either before or after planting, i.e., pre-plant-pre-emergence; post-plant-post-emergence.

4.4.3 Spraying or spreading on foliage post-emergence.

4.5 Treatment dosages.- Greenhouse and growth chamber testing usually provides a basis for field test dosage selection. In all instances a control should be included. The dosages should range from the "no effect" level to rather severe drastic plant damage so that a full range of plant responses may be observed.

4.5.1 Field plot soil amendment may be calculated on a volumetric basis with either overall amendment of the test plot area to a 15-cm depth or band amendment in a band 50 cm wide by 15 cm deep. Normally the test substance, in a finely divided state, is evenly deposited on the soil surface and then "rototilled" into the profile followed by planting in the center of the band. Depending on previous experience, the test dosages may range from 0.1 μg/ml to 1000 μg/ml soil volume to $\log_{10}$ progression volumetric basis.

4.5.2 Spray or granule treatments may be made to the soil surface post-seeding but pre-emergence of seedlings. Treatment may be made overall to emerged foliage as well as exposed soil, or only to foliage. Dosages in this instance are normally calculated on the basis of surface area treated. Applications may be made to seedlings, mature plants, singly or repetitively depending on the purpose of the test. A possible dosage range would be from 0.1 kg per hectare to 1000 kg per hectare equivalent. The logarithmic dosage technique is one of the best ways of achieving a wide range of response. (7.3.4)

4.6 Application equipment.- The major criterion for application equipment is that the substance is applied uniformly and reproducibly at the dosage or range of dosages desired. Sprayers, dusters, or granule spreaders may be used.

4.6.1 Granule spreaders of normal commercial use are satisfactory if properly calibrated.

4.6.2 Many types of sprayers have been developed for herbicide evaluation. (7.3.1 to 7.3.12)

5. DATA REPORTING--EFFECTS EVALUATION.

5.1 Potential effects are listed below. An asterisk denotes those effects that should be determined in all instances.

5.1.1 Visual changes in morphology.

5.1.1.1 Above ground

a. foliage*- leaves, petioles, foliar arrangements-size, shape, distortion, color.

b. support structure*- stems, limbs, stolons, tillers, -size, shape, distortion, color.

c. reproductive organs*- flowers, fruits, seeds-shape, distortion, color.

5.1.1.2 Changes not visually detectable

a. rhizomes, tubers, corms, bulbs- size, shape distortion, color.

b. roots*- size, shape, distortion, color.

5.1.2 Below ground.

5.1.2.1 Anatomical- - tissue and cellular arrangement.

5.1.2.2 Physiological.

a. Chemical constituents.

1. nutrients, vitamins, etc.
2. metallic and non-metallic elements.
3. other organic and inorganic constituents.

b. Crop plant yields or other subtle changes in morphology which are not visually detectable but only discernible through quantitative measurements* ,i.e., yield*, height*, weight*, etc.

c. Changes in reproduction.

1. mutations
2. progeny abnormalities in F_1 populations

6. DATA INTERPRETATION.

Appropriate statistical analysis or quantitative information obtained should be made.

6.1 Perhaps the statistical method best adapted to the study of results from field plots is the analysis of variance. (7.11, 7.12) Certain aspects of the analysis of variance should be considered:

6.1.1 The analysis of variance enables a researcher to test the significance of the observed results of the experiment. The objective is to obtain a variance for the observed effects, such as treatment differences, and a variance for error. The ratio of these variances will be equal to one if there are no effects owing to the treatments. If the ratio is greater than one, no significance can be attached to the results. When the ratio is equal to or greater than its 5 percent point it can be concluded that varietal differences are real.

6.1.2 Replicates increase the precision of an experiment by controlling error. The error variance results from differences between plots that are alike. When replicates are used, each treatment appears in each replicate. In such experiments the differences between the plots of any one variety are due to experimental error, the average differences between the replicates. Therefore, the replicate variance is removed from the error. The larger the proportion of the total variability that is removed, the more accurate the experiment.

6.2 If the anticipated exposure level of the test substance in situ will reach 0.01 of the levels that result in significant effects during field testing, then the release level of the substance in actual manufacture should either be reduced to a satisfactory level or the release prevented from taking place. Long-term effects from low-level chronic doses presents a problem which requires long term testing with multi-exposure or continuous exposure techniques. The procedures are beyond the scope of this outline.

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7.3 Field plot sprayers.

7.3.1 Derscheid, L. A. Sprayers for use on experimental plots. *Weeds* 1: 329-337; 1951.

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3.8 ADDITIONAL LISTINGS OF TEST PROCEDURES FOR TERRESTRIAL TRACHEOPHYTA FOR LABORATORY AND GREENHOUSE

Terrestrial vascular plants form the basis for the earth's crops for human and animal food and for support of a multitude of other organisms. In addition, through the photosynthetic processes conducted by these plants a major portion of the earth's organic molecules are produced and transformed either directly or indirectly. However, the effect of soil and water pollutants is almost unknown. The available knowledge is is restricted to a few crop plants with little information about native plant communities.

Our search of the world's literature produced no information regarding test procedures or investigations with the Bryophyta. Among the Tracheophyta no research has been conducted with the Lycopsida, Pteropsida, or Sphenopsida. The bulk of the available information pertained to crop plants and "weed" plants within the Angiospermae and Coniferophyta.

As noted previously, test procedures may be performed in the laboratory, growth chambers, greenhouses, fields, or with entire plant communities in nature. Laboratory incubator, growth chamber and greenhouse procedures can be performed at any time of the year with results obtained in the shortest interval. However, the correlation between small pot, petri dish, tube, sand, or liquid culture test results and field or natural plant communities results may be very poor, especially if the effects are exerted primarily or exclusively on the mature plant or its reproduction. The soil and air environment exert major effects on test results. Some substances may be toxic only under environmental stress.

Plant species are extremely variable in response to different toxicants. Due to the lack of knowledge of chemical effects (except for the Spermatophyta), it is strongly recommended that initial testing be restricted to the monocotyledoneae and dicotyledoneae. In later secondary test procedures Coniferophyta may be appropriate under circumstances where substances will come in direct contact with coniferous vegetation. Crop plants should be employed because the information available concerning species and cultivar variability, environmental and toxic responses, and overall physiology is far greater than for any wild plants regardless of genera or species. The significance and importance of crop plant response is much easier to grasp than with unknown wild species.

Laboratory, incubator, growth chamber, and greenhouse procedures

1. This procedure represents a general sequence of testing beginning with seed germination and growth chamber studies of seeds surrounded by toxicant soaked filter paper proceeding to the germination and growth of seeds in toxicant treated soil. It can employ chemicals with various modes

of action and a number of crop species.

Chang, In-kook, and C. L. Foy Effect of picloram on germination and seedling development of four species. *Weed Sci.* **19: 58-64; 1971.**

2. A sequence of three bioassays is proposed which will provide a broad spectrum of sensitivity to varying modes of toxic action. These procedures consist of a seedling root elongation, shoot elongation, and a *Chlorella* chlorophyll extraction. These can be conducted in the growth chamber.

Kratky, B. A. and G. F. Warren. The use of three simple rapid bio-assays on fourth-two herbicides. *Weed Res.* 11: 257-262; 1971.

3. This is a rapid method which should detect toxicants that inhibit photosynthesis.

Parker, C. A rapid bio-assay method for the detection of herbicides which inhibit photosynthesis. *Weed Res.* 5: 181-184; 1965.

4. Growth chamber procedures involving seed treatment, sprouted seeds and seedling exposure in nutrient solutions are presented. Injury is expressed in terms of MD_{50} (a molarity dosage which causes 50% kill or inhibition of the organism tested).

Sund, K. A. and N. Nomura. Laboratory evaluation of several herbicides. *Weed Res.* 3: 35-43; 1963.

5. This technique provides a measure of toxicant injury to root growth as well as evaluating translocation potential.

Gentner, W. A. A technique to assay herbicide translocation and its effect on root growth. *Weed Sci.* 18: 715-716; 1970.

6. This procedure, although primarily developed to quantify herbicide residues by bioassay, may be used to measure toxicant effects through soil exposure routes. This method stresses uniformity of procedure especially soil and air environments.

Santelmann, P. W., J. B. Weber, and A. F. Wiese. A study of soil bioassay technique using prometryne. *Weed Sci.* 19: 170-174; 1970.

7. Rapid techniques are described for a foliage and three complementary soil bioassays to measure the presence of toxicants from various exposure routes. This series should be effective in measuring phytotoxicity from exposure to a wide range of toxicants as well as the pichloram employed in the report.

Leasure, J. K. Bioassay methods for 4-amino-3,4,4-trichloropicolinic acid. *Weed Sci.* 12: 232-234; 1964.

8-9 These papers describe a growth chamber procedure to evaluate the effects of toxicants on seed germination and seedling growth including morphological and anatomical modifications of shoots and roots. Very little space is required for these tests.

Cutter, Elizabeth, F. M. Ashton, and Donna Huffstutter. The effects of bensulide on the growth, morphology, and anatomy of oat roots. *Weed Res.* 8: 346-352; 1968.

Ashton, F. M., Elizabeth G. Cutter, and Donna Huffstutter. Growth and structural modifications of oats induced by Bromacil. *Weed Res.* 9: 198-204; 1969.

10. This unique approach to bioassay involves soil-toxicant mixtures in petri dishes in which seeds are germinated. In addition, standard soil cup-seedling growth procedures are described for toxicant incorporation into soil. Although the petri dish technique required only an incubator, the short duration would not detect photosynthesis inhibiting compounds.

Horowitz, M. and Nira Hulin. A rapid bioassay for diphenamid and its application in soil studies. *Weed Res.* 11:143-149; 1971.

11. This petri dish technique employs pre-germinated seeds with dishes placed in vertical position during incubation. Root and shoot elongation inhibition may be determined. Variables may be incorporated into the experiment to include such things as soil moisture levels, adsorption, soil texture, etc. The short duration would not be effective in detecting photosynthesis inhibitors.

Horowitz M. A rapid bioassay for PEBC and its application in volatilization and adsorption studies. *Weed Res.* 6: 22-36; 1966.

12. Although twenty-five years old, the series of procedures detailed in this paper still provide a good basis for detection of phytotoxicity. Growth chamber or greenhouse techniques with preemergence soil surface sprays as well as pre-and post emergence field plot sprays are included.

Shaw, W. D. and C. R. Swanson. Techniques and equipment used evaluating chemicals for their herbicidal properties. *Weeds* 1: 352-365; 1951

3.9 ADDITIONAL LISTINGS OF TEST PROCEDURES TO DETERMINE INFLUENCE OF ENVIRONMENT ON PHYTOTOXICITY IN LABORATORY, GREENHOUSE AND FIELD.

As evident from previous discussions, many environmental and treatment factors may influence toxicity by test substances. When any initial screening procedure in laboratory incubator, greenhouse or growth chamber indicates potential phytotoxicity the next essential step is elucidation of the influence, both exposure factors and the environment, on symptom expression. The following must be evaluated:

1. Exposure factors-spray droplets size.
2. Environmental factors.
    a. Climatic
        (1) temperature
        (2) humidity
        (3) light
    b. Edaphic
        (1) soil pH
        (2) nutrients
        (3) texture
        (4) temperature
        (5) organic matter.

The acceptable procedures available to determine these factors are listed below by categories.

3.9.1 Influence of Spray Droplet Size

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3.9.5 Vapor Transfer from Soil

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3.9.6 Persistence and Movement in Soil

Eshel, Y. and G. F. Warren. A simplified method for determining phytoxicity, leaching and adsorption of herbicides in soils. *Weeds* 15:115-118; 1967.

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3.10 ADDITIONAL LISTINGS OF SPRAY APPLICATION EQUIPMENT FOR PHYTOTOXICANTS IN GROWTH CHAMBERS, GREENHOUSE AND FIELD

3.10.1 Growth Chamber and Greenhouse

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Webster, D. H. and J. S. Leefe. A small plot sprayer using disposable spray containers. *Weeds* 9:323-324; 1961.

3.11 MEASUREMENT OF PHYTOTOXICITY USING AQUATIC PLANTS

3.11.1 Introduction

Stephan A. Forbes, in his now classic 1887 paper titled, "The Lake as a Microcosm," noted the great interdependence within aquatic ecosystems. The freedom of one organism from the influence of another is often much more restricted than in many terrestrial systems. As an example, metabolites released by terrestrial organisms are generally more spatially limited in effect than are the readily diffusible materials within the aquatic medium. However, since aquatic zones have not been utilized for agricultural purposes, as have terrestrial regions, the aquatic community had historically been treated as a source of fish and other readily apparent members of the top of the fiid chain, rather than as a highly integrated 'microcosm.'

In recent years, however, a wise shift has been taking place as the primary producers of aquatic systems have received increased attention. Part of the emphasis has been due to the adverse effects of nuisance plant growth on fish life, but an increasing amount of it has hopefully been undertaken with a view of the basic importance of aquatic plants to both aquatic and terrestrial life.

Numerous efforts have been put forth to establish standardized testing procedures for aquatic plants, but no one protocol has been found to be applicable to all situations. As noted for terrestrial plant testing, plant species, culture age, dosage, chemical formulation, and other factors greatly influence test results. Specific influences in the aquatic environment include solubility, degradation and deactivation potential, method of release into the ecosystem, dilution, and chemical characteristics of the receiving water.

Certainly a screening test using either algae or aquatic vascular plants will not produce a complete answer to material toxicity within the aquatic environment. Such tests will, however, demonstrate some effects of specified materials upon some basic members or representatives of the aquatic ecosystem. The extent of testing and the species used would necessarily be dependent on a variety of factors relative to the nature of the material in question and the receiving water system.

3.11.2 Algae

The use of algae as test organisms is well established in plant physiology. Their use as bioassay organisms has been much more limited, however, and thus techniques have not been generally standardized. Except for relatively recent attempts coordinated by T. E. Maloney (Algal Assay Procedure, 1971), few efforts to develop a sizeable data base upon which to build a standard protocol have obtained any broad acceptance. Many of these attempts have viewed algae principally as potential nuisance organisms in eutrophication rather than as bioassay test organisms in their own right.

Methods of measuring effects of chemicals on algae:

A wide variety of procedures are available to measure alterations in algal activities. The following list includes some of the reported approaches:

1) oxygen evolution rate
2) carbon-14 uptake rate
3) increase or decrease in cell numbers
4) increase or decrease in cell size
5) chlorophyll-a content
6) chlorophyll-carotenoid ratio
7) nitrogenase activity
8) heterocyst frequency
9) akinete development
10) biomass changes
11) ATP content
12) cell division rate
13) plant composition variations
14) optical density of cultures
15) community structure
16) diversity estimates

The choice of approach reported in much of the literature has obviously been strongly influenced by instrumentation availability, taxonomic capability, and interest of the investigator. Only in a relatively few instances have algal bioassay investigators reported adequate preliminary research to determine the method or methods most applicable to the situation. As a result, workers have chosen their own light quality and quantity, evaluation of effect procedure, duration of run, specie of organism, growth medium, and any of numerous other design features.

No single species of aquatic plant (vascular or algae) and no single test procedure can fully evaluate the toxic capabilities of any given material. The addition of an aquatic plant early in any overall test regime will, however, greatly enhance the potential to evaluate possible phytotoxicity more completely. Since most bioassay procedures use single species cultures, great care must be exercised in extrapolating information derived from such tests to the environment in general.

The first factor in determining the extent of algal testing is the expected quantity and area of environmental release. The lack of direct aquatic release does not diminish the efficacy of algal bioassays. With the proper choice of organisms, the test results are equally applicable to the evaluation of effects on soil algae. Due to the flowing nature of many potential receiving waters, no direct aquatic discharge can be considered as a strictly local application. Therefore, even relatively small quantities of materials to be released into the aquatic environment should have some algal bioassay testing.

The chemical nature of the material to be released is an important consideration. Information concerning biodegradibility, nutrient potential, or other possible effects may be determined ahead of time. In each case, it must be recognized that different environments react differently to a given effluent.

The actual method of waste handling, product disposal, or other method by which material may reach the aquatic system will also require consideration when determining the most appropriate testing program. To paraphase a common cliche, the dilution factor should not be considered as a solution to pollution. Nevertheless, such factors are important in establishing a basis for appropriate testing. As an example, in the absence of biological concentration mechanisms, materials should be worked with in concentrations approximating those expected in discharges rather than at concentrations several orders of magnitude higher.

Two major categories of test materials emerge from these considerations: 1) pure compounds and 2) mixed or composite effluents. Bioassays using materials from the first category are relatively easy to work with. Tests are repeatable and analytical work is simplified. Materials in the second category, however, impose a different set of considerations. It is at this point that the bottomless pit opens, as daily variations in effluents, synergistic reactions, season, photochemical processes, and innumerable other factors enter in. Obviously, to examine the effects of all such possible combinations of factors becomes prohibitive in both time and resources. Therefore, rather than test for all possible effects, a feedback system is recommended that allows retesting of materials using different regimes as evidence points to such a need.

In general, algal bioassays are divided into two major approaches, laboratory (in vitro) and field (in situ). Laboratory bioassays are further subdivided into static and flow-through designs. Field bioassays may be subdivided into open-system designs, such as ponds, and closed system bottle designs.

Static in vitro tests are the most commonly used form of algal bioassay, but continuous flow designs applicable to certain analysis have recently been further developed. To be generally applicable for the purposes of this report, however, the key requirements for test fitness listed in the PAAP must be considered

1) They should be so designed that technician-level personnel can do them .

2) Equipment and instrumentation requirements should be relatively modest and readily attainable .

3) The procedures should be so standardized that results are acceptably reproducible .

4) Geographic location should not affect the test results .

5) The results can be applied with judgment to field conditions.

A central point in acheiving reproducible algal bioassays is the use of proper media. Available formulae range from highly defined chemical mixtures to indeterminate soil extracts. The use of an applicable defined medium is considered to be a necessary beginning point, even though undefined media, such as those using prepared effluent receiving water, may be more appropriate during final testing stages.

The selection of test species for either static or continuous flow bioassays should be based on a variety of criteria, among them the following: (1) general availability of standard cultures; (2) similarity of test species to locally known flora; and (3) knowledge of the organism's physiology;and (4) suitability of the organism for routine culturing in defined media.

The selection of species for flow-through apparatus is further restricted to organisms that grow attached to a substrate or are otherwise suitable for such applications. Greater technical capabilities are probably needed for continuous-flow operations than for static testing.

Large scale pond type in situ testing requires fairly large areas, extensive equipment, and considerable expertise for proper evaluation. As a result, this type of algal testing is more research oriented than bioassay directed. In situ bottle tests, on the other hand, are well established as an evaluation method and are highly applicable to some testing situations. Carbon uptake rates, using carbon-14 or other physiological rate measurements, are frequently used to assess a variety of effects of environmental releases of materials.

Acute phytotoxicity tests, using algae, can be completed in relatively short time periods, ranging from a few hours to a few days. Methods of determining chronic effects are, however, poorly developed for general application and interpretation of results is, at at this time, questionable.

The appropriate applications of algal bioassays are many and varied. Inclusion of one or two algae in the earliest screening seems appropriate both from an information and a cost point of view. A static algal bioassay during screening is fairly inexpensive and straight forward interpretation is possible with applications to both terrestrial and aquatic systems. As the need to test a given material increases so can the complexity and effectiveness of algal testing increase.

3.11.3 Vascular Plants

According to the literature and interviews conducted for this project, the major use of aquatic vascular plants has been to evaluate herbicidal qualities of chemicals. This type of activity, though informative, has not resulted in a published information based wholly applicable to routine bioassay of low-level phytotoxic effects.

Methods are appended for use of aquatic vascular plants in bioassay, but considerably more work needs to be published and standardized before wholesale adoption of such tests in routine practice.

3.12 PROCEDURE FOR PRELIMINARY EVALUATION OF POTENTIAL TOXICITY TO ALGAE THROUGH LABORATORY BIOASSAY TESTING. (Adapted from the Provisional Algal Assay Procedure -EPA), (PAAP)

1. SCOPE

1.1 These procedures are intended to provide a basis for evaluating the effects of a variety of chemical compounds and/or mixtures on the growth and death rate of cultured algae. The procedures are suitable for determining gross effects and are not intended for use in detailed physiological or biochemical studies.

2. SIGNIFICANCE

2.1 Significant effects observed in these tests point to the need for more refined approaches to ellucidate probable modes of action, since coagulation, nutrient binding, and other indirect causes may result in apparent toxicity.

3. EQUIPMENT AND FACILITIES

3.1 General laboratory facilities suitable for algal culturing and growth measurements.

3.1.1 Culturing and incubation facilities - either a temperature stable room or an incubator.

3.1.2 Culture vessels - good quality Erlenmeyer flasks such as Pyrex or Kimax. For uniform light transmission, etc., the same brand should be used within a laboratory. To achieve optimum surface to volume ratios for adequate carbon dioxide transfer, the following ratios are suggested:

(a) 40-50 ml liquid in 125 ml flasks
(b) 60-80 ml liquid in 250 ml flasks
(c) 100-130 ml liquid in 500 ml flasks

3.1.3 Culture closures of foam, gauze, or other material that permits adequate gas exchange but prevents contamination.

3.1.4 Lighting facilities to provide equal illumination to all flasks. "Cool White" or similar type fluorescent illumination should be able to provide between 200 and 800 ft-c.

3.1.5 Light meter to measure foot-candles or use photographic light meter and convert to ft-c according to formula given elsewhere in this paper.

3.1.6 Microscope of good quality and up to 400x magnification.

3.1.7 Counting chamber - Palmer cell, hemacytometer, or similar type cell. The Sedgewick-Rafter chamber is generally unacceptable for algal identification and ennumeration.

3.1.8 pH meter accurate within ±0.1 units.

3.1.9 Spectrophotometer for use between 600 and 750 nm (optional).

3.1.10 Coulter electronic cell counter (optional).

3.1.11 Fluorometer (optional).

3.1.12 Shaker capable of approximately 100 cycles/minute (optional).

4. TEST ORGANISMS

4.1 Fresh water (for source cultures see 7.7)

4.1.1 *Selenastrum capricornutum* Printz. (green alga)

4.1.2 *Anacystis cyanea* Drouet and Dailey (blue-green alga) (formerly *Microcystis aeruginosa* Kutz. emend Elenkin)

4.1.3 *Anabaena flos-aquae* (Lyngb.) DeBrebisson (blue-green alga)

4.1.4 Other fresh water algae with similar background in bioassay or physiological studies may be considered equally appropriate.

4.2 Marine

4.2.1 *Dunaliella tertiolecta* Butcher (green flagellate alga)

4.2.2 *Thalassiosira pseudonana* Hasle and Heimdal (diatom alga)

4.2.3 Other marine or brackish water algae with similar background in bioassay of physiological studies may be considered equally appropriate.

4.3 Test species, if maintained in the laboratory, should be kept under conditions that will insure stability and similarity of the isolate over time to prevent changes in response pattern to chemical exposure.

5. TEST PROCEDURES

5.1 Culture medium (fresh water)

5.1.1 Composition (macronutrients)

Compound	Final Concentration (mg/l)	Quantity to make 1 liter stock solution at 1000x
$NaNO_3$	25.500	25.500 g
K_2HPO_4	1.055	1.044 g
$MgCl_2$	5.700	5.700 g
$MgSO_4 \cdot 7H_2O$	14.700	14.700 g
$CaCl_2 \cdot 2H_2O$	4.410	4.410 g
$NaHCO_3$	15.000	15.000 g
(micronutrients)	(μg/l)	
H_3BO_3	185.520	0.185520 g
$MnCl_2$	264.264	0.264264 g
$ZnCl_2$	32.709	0.032709 g
$CoCl_2$	0.780	0.000780 g

$CuCl_2$	0.009	0.000009	g
$Na_2MoO4 \cdot 2H_2O$	7.260	0.007260	g
$FeCl_3$	96.000	0.096000	g
$NaEDTA \cdot 2H_2O$	300.000	0.30000	g

5.1.2 Element concentrations

Element	Concentration (mg/l)	Element	Concentration (μg/ml)
N	4.200	B	32.460
P	0.186	Mn	115.374
Mg	2.904	Zn	15.691
S	1.911	Co	0.354
C	2.143	Cu	0.004
Ca	1.202	Mo	2.878
Na	11.001	Fe	33.051
K	0.469		

5.1.3 Stock solutions of 1000 times final concentration are used by combining 1 ml of each solution into a final volume of 1 liter distilled water (glass-distilled is preferred).

5.1.4 Autoclaving of final solutions should be at 15 psi ($1.1\ kg/cm^3$) at 121°C for 10-15 minutes. Adjust volume following autoclaving. If an electric particle counter is to be used for counting cells, filter and autoclave solution through a 0.45μ membrane filter.

5.1.5 If the test organism is a diatom, add the following to the culture medium:

Compound	Concentration (mg/l)	gm/l in stock	Element	Concentration (mg/l)
$Na_2SiO_3 \cdot 7H_2O$	101.214	101.214 gm	Si	9.980

5.2 Culture medium (marine)

5.2.1 Seawater media vary accoring to the organism and other factors. Proper selection of ingredients, including micronutrients and vitamins will need to be made in accord with test requirements. The media of Guillard and Ryther (1962), Woods Hole modifications of Guillard and Ryther, the Marine Algal Assay Procedure: Bottle Test, and numerous others are available.

5.3 Algal inoculum

5.3.1 Cultures, up to three weeks old may be used as a source of inoculum. For *Selenastrum* and most other green algae and diatoms, one-week incubation is often suffiicient to provide enough cells. Two to three weeks may be required to provide inocula for assays with blue-green species.

5.3.2 The inoculum may be used directly from stock cultures or the cells may be centrifuged. The sedimented cells should then be resuspended in an appropriate volume of distilled water containing 15 mg $NaHCO_3$/l and again centrifuged. The sedimented algae should again be resuspended in the water-bicarbonate solution and used as the inoculum. Centrifugation should not exceed either 1000 rpm or 10 minutes.

5.4 Incubation

5.4.1 Incubation should be light as described, with the illumination measured adjacent to the flask at liquid level. Temperature should remain constant (± 2°C).

5.4.2 To prevent excessive cell death due to settling, the cultures should be incubated on a shaker at approximately 100 cycles per minute or swirled by hand, on a regular schedule, at least twice a day. Shaker incubation has the added advantage of increasing CO_2 exchange.

6. TEST EVALUATION

6.1 Growth parameters

6.1.1 Maximum specific growth rate

6.1.1.1 The maximum growth rate (μ_{max}) for an individual flask is the largest specific growth rate (μ) occurring at any time during incubation. The μ_{max} for a set of replicate flasks is determined by averaging μ_{max} of the individual flasks.

The specific growth rate, μ, is defined by

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1} \text{ days}^{-1}$$

where X_2 =biomass concentration at end of selected time interval

X_1 =biomass concentration at beginning of selected time interval

$t_2 - t_1$ =elapsed time (in days) between selected determinations of biomass

NOTE: IF BIOMASS (DRY WEIGHT) IS DETERMINED INDIRECTLY, E.G., BY CELL COUNTS, THE SPECIFIC GROWTH RATE MAY BE COMPUTED DIRECTLY FROM THESE DETERMINATIONS WITHOUT CONVERSION TO BIOMASS, PROVIDED THE FACTOR RELATING THE INDIRECT DETERMINATION TO BIOMASS REMAINS CONSTANT FOR THE PERIOD CONSIDERED.

6.1.1.2 Laboratory measurements - The specific growth rate occurs during the logarithmic phase of growth - usually between day 0 and day 5 - and therefore it is necessary that measurements of biomass be made at least during the first 5 days of incubation to determine this maximum rate. Indirect measurements of biomass, such as cell counts, will normally be required because of the difficulty in making accurate gravimetric measurements are made should be recorded for use in the compoutations.

6.1.1.3 Computation of maximum specific growth rate - The maximum specific growth rate (μ_{max}) can be determined by calculation using the equation in 6.1.1.1 to determine the daily specific growth rate (μ) for each replicate flask and averaging the largest value for each flask. It may also be determined by preparing a semi-log plot of biomass concentration versus time for each replicate flask. Ideally, the exponential growth phase can be identified by 3 or 4 points which lie on a straight line on this plot. However, the data often deviate somewhat from a straight line, so a line judged to approximate most closely the exponential growth phase is drawn on the plot. If it appears that the data described two straight lines, the line of steepest slope should be used. A linear regression analysis of the data may also be used to determine the best fit straight line. Two data points that most closely fit the line are selected and the specific growth rate (μ) is determined according to the equation given in 6.1.1.1. The largest specific growth rates for the replicate flasks are averaged to obtain μ_{max}.

6.1.2 Maximum standing crop

6.1.2.1 Definition - The maximum standing crop in any flask is defined as the maximum algal biomass achieved during incubation. For practical purposes, it may be assumed that the maximum standing crop has been achieved when the increase in biomass is less than 5 percent per day.

6.1.2.2 Laboratory measurement - After the maximum standing crop has been achieved, the dry weight of algal biomass may be determined gravimetrically using either the aluminum-dish or filteration technique. If biomass is determined indirectly, the results should be converted to an equivalent dry weight using appropriate conversion factors.

6.1.3 Biomass monitoring - several methods may be used, but they must always be related to dry weight.

6.1.3.1 Dry Weight - gravimetrically

6.1.3.2 By direct microscopic counting (appropriate counting cell) or the use of an electronic particle counter. *Anabaena flos-aquae*, or other filamentous forms, are not amendable to counting with an electronic particle counter. Microscopic counting can be facilitated by breaking up the algal filaments with a high speed blender or by sonication.

6.1.3.3 Absorbance - with a spectrophotometer or colorimeter at a wavelength of 600-750 nm. In reporting the results, the instrument make or model, the geometry and path length of the cuvette, the wave length used, and the equivalence to biomass should be reported.

6.1.3.4 Chlorophyll - after extraction or by direct fluorometric determination. The equivalence between chlorophyll content and biomass should be reported.

6.1.3.5 Total cell carbon - by carbon analyzer. Equivalence between total cell carbon and biomass should be reported.

6.2 Data analysis

6.2.1 The principal measures of the culture activity are:dry weight, cell numbers, pigment content, or other biomass indicators as previously noted. It is usually appropriate to include experimentally determined conversion factors between the indicator used and the dry weight. More than one growth or biomass indicator should be used whenever possible.

6.2.2 The overall evaluation of algal bioassay results consists of two parts. The first is the determination of whether a given set of results is significant when considered as a laboratory measurement. Several methods are available such as Student's t- test and analysis of variance. (A sufficient number or replicates is therefore necessary for statistical analysis.) It must be emphasized, however, that no set criteria presently exist to determine what level response is significant. Each evaluation must be conducted on the basis of specific test objectives using valid statistical procedures. (Some laboratories do not use set evaluative routines, such as an equivalent to an LC_{50}) The second part of the overall evaluation is the correlation of laboratory bioassay results with those observed or predicted in the field. No specific guidelines are yet available for this purpose.

7. REFERENCES

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3.13 APPROACH FOR THE EVALUATION OF TOXICITY TO AQUATIC VASCULAR PLANTS

1. SCOPE

1.1 This procedure is intended to provide guidelines for those who wish to use aquatic vascular plants in the screening of potentially phytotoxic materials. It is not intended as a definitive test, but will provide an indication of gross effects on vascular plants in an aquatic environment.

2. SIGNIFICANCE

2.1 High levels of toxicity exhibited by a material in this procedure indicates the need for further testing in those instances where aquatic or run-off release is anticipated.

3. EQUIPMENT AND FACILITIES

3.1 General laboratory facilities suitable for culturing and maintaining small aquatic vascular plants.

3.1.1 Temperature stable room or incubator.

3.1.2 Culture vessels - Erlenmeyer flasks or other suitable containers. For uniform light transmission, the same brand should be used within any given experimental run. Flask sizes up to 1500 ml, test tubes 175 x 20 mm, etc.

3.1.3 Culture flask closures of foam, gauze, or other material that permits adequate gas exchange but prevents contamination.

3.1.4 Lighting of the "Cool White" fluorescent type to provide equal illumination to all flasks of between 200 and 400 ft-c.

3.1.5 Light meter to measure ft-c or use photographic light meter and convert to ft-c according to formula given elsewhere in this paper.

4. TEST ORGANISMS

4.1 *Lemna gibba* L., *Lemna minor* L., or others.

4.2 *Cabomba caroliniana* Gray.

4.3 *Elodea canadensis* Rich. in Michx.

4.4 Other small aquatic vascular plants may be equally suitable.

5. TEST PROCEDURES

5.1 Culture medium (modified Hoagland's solution)

Compound	Stock Solution (g/1)	cc of stock to make one liter of nutrient solution (cc/1)
$Ca(NO_3)_2 \cdot 4H_2O$	118.08	10
KNO_3	50.25	10
$MgSO_4 \cdot 7H_2O$	24.08	10
KH_2PO_4	13.61	10
the following (except EDTA and Fe) to be combined into one stock		
H_3BO_3	2.860	
$MnCl_2 \cdot 4H_2O$	1.810	
or $MnSO_4 \cdot H_2O$	1.540	

Compound	Stock Solution (g/l)	cc of stock to make one liter of nutrient solution (cc/l)
$ZnSO_4 \cdot 7H_2O$	0.220	1
$CuSO_4 \cdot 5H_2O$	0.080	
$H_2MoO_4 \cdot H_2O$	0.090	
or $MoO_3 \cdot H_2O$	0.075	
EDTA (potassium salt)	2.500	1
$FeSO_4 \cdot 7H_2O$	2.500	

(It may be desirable to add 10 grams per liter sucrose to the nutrient medium of some plants.)

5.2 Nutrient solutions should be autoclaved in stoppered containers at 15 psi (1. 1 kg-cm^3) at 121°C for 15 minutes.

5.3 Sterile cultures (if needed to avoid interferences)

5.3.1 Immerse in 0.1% $HgCl_2$ for 45 seconds and rinse in sterile water.

5.3.2 Immerse in 50% ethyl alcohol for 30 seconds and rinse twice in sterile nutrient.

5.3.3 Place sterile plants in sterile culture flasks where regrowth will develop sterile cultures.

5.3.4 Transfer plants every week to maintain appropriate growth conditions and to prevent crowding.

5.4 Three to ten plants or plant tips, depending on species, are used in testing materials. At least duplicate tests at appropriate concentrations should be run for 1-3 weeks.

6. TEST EVALUATION

6.1 The effect is evaluated by comparing appearance of test plants with control plants grown in nutrient solution.

7. REFERENCES

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3.14 EVALUATION OF THE PHYTOTOXICITY OF AIRBORNE SUBSTANCES

One can formulate a possible strategy for the regulation of airborne emissions of potential toxic substances. The strategy outlined below takes into account only direct effects on plants, but indirect effects such as the alteration by a toxicant of disease susceptibility; the plant as an accumulator of a toxicant and the means by which it can be transferred to other components of the biotic environment; or subtle effects of a toxicant on plant growth and form, yield or quality are not considered. The strategy also includes two phases: (1) an initial screening for compounds that will have limited production and (2) a secondary screening that will require establishment of the threshold for plant injury.

1. Initial Screening

The purpose of the initial screening is to establish the relative toxicity to selected plant species of the chemical to be released.

1.1 Plant Materials - Because it will be necessary to conduct tests on phytotoxicity of chemicals in different parts of the United States, reliance solely on dominant species of native vegetation especially for initial screening may be impractical for several reasons: 1) they may be difficult to transplant or grow, 2) they may be slow growing,

3) they may be difficult to evaluate with respect to injury. It would be simpler and more practical to select plants that grow rapidly and uniformly, inexpensive to produce in large quantities, relatively susceptible to some known toxicants, and are representative of large groups of plants. These criteria for initial screening can be met by the use of a cultivar of the common bean (*Phaseolus vulgaris* L.) and corn (*Zea mays* L.). Bean, a dicotyledonous plant, is representative of the broad-leaved plants and corn, a monocotyledonous plant, of the grains and grasses. For purposes of uniformity and simplicity, plants could be grown in 4-inch pots in a synthetic medium which can be reproduced easily. Any of the peat-perlite, peat-vermiculite, or other available mixes can be used. Six seeds should be sown in each pot and, after emergence, the seedlings should be thinned to three per pot. Each fumigation should consist of at least 6 pots of each species (18 plants).

1.2 Fumigation Equipment The fumigation chambers used should have the following characteristics: 1) they should be of a size to accommodate a sufficient number of replicates of the species under test; 2) they should have an air delivery system capable of exchanging the chamber air about once each minute; 3) there should be an inlet into the air delivery system for introduction of the chemical being tested; 4) the air delivery system should be designed in such a way as to give even distribution through the chamber of the chemical being tested. The air supply for the chambers should pass through a particulate filter and through another filter to remove ambient phytotoxicants other than that being tested. The most common and universally distributed ambient phytotoxicant is ozone and it can be removed by passing air through an activated charcoal filter. A satisfactory chamber for the purpose has been described by Heck et al. (1,2).

Appropriate devices must be adapted or developed to meter the test chemical into the treatment chamber, to maintain desired concentrations, and to monitor the concentration of the chemical during the exposure period. No single device can be recommended which is satisfactory for all substances under all conditions; but a commonly used apparatus is the Greenburg-Smith impinger (3,4). The Greenburg-Smith impinger is based upon the impingement of a gas or particle-containing stream in an appropriate liquid medium. After the sampling period, an analysis is made for the substance of interest.

1.3 Fumigation Procedures

1.3.1. Concentration - There is no completely satisfactory method to estimate the concentration of the test substance to be used, but it would seem to be an unnecessary requirement at this stage of testing to attempt to establish the threshold dose for plant injury. Therefore, for purposes of initial screening, one concentration might be used and it might be established as five times the highest predicted ambient concentration (at the property line) based upon estimated losses to the atmosphere and local meteorological and typographical conditions.

1.3.2. Duration - A four-hour exposure period is recommended.

1.3.3. Test Conditions - No specific parameters can be required except that tests should be carried out at temperatures and relative humidities as near as possible to those found under ambient conditions during midsummer. Fumigations should always be made under light conditions of at least 1500 ft-c .

1.3.4. Plant Evaluation - Any foliar lesion produced by exposure to the test substance should be considered as a positive effect.

1.4 Results

1.4.1. No Effect - If no visible change has occurred on the plant 48 hours after exposure, the assumption is made that the test substance is safe for limited production.

1.4.2. Effect - If visible lesions have been induced on the plant 48 hours after exposure, manufacture is restricted or a more adequate atmospheric control system will be required.

2 SECONDARY SCREENING

The purpose of the secondary screening is to establish the threshold of the chemical for plant injury when (1) the chemical is to be produced in large quantities or (2) if the chemical fails to pass the initial screening.

2.1. Plant Materials - The same species recommended for initial screening should be used also for secondary screening. Where practicable, consideration should also be given for testing of easily cultivated native species, both woody and herbaceous. Adequate replication should be included in each fumigation.

2.2 Fumigation Equipment - The same chambers recommended for initial screening are suitable for these tests.

2.3 Fumigation Procedures

2.3.1. Concentration - One approach to the determination of the threshold for plant injury is estimation by up-and-down techniques, one of which is the staircase method of Finney (5). By this method, the ED_{50} (effective dose at which 50% of the subjects respond) is estimated from the data acquired. In the staircase method, a series of equally spaced log doses is chosen, e.g., $\ldots, x_{-3}, x_{-2}, x_{-1}, x_0, x_1, x_2, \ldots$, where x_0 is believed to be near the log ED_{50}. The initial screening may be useful for this estimation. The first group of plants is tested at X_0. Thereafter, the result of any test determines the dose for the next test: if an effect is produced, the next test is conducted at a dose one step lower; if an effect is not produced, the next test is at a dose one step higher. Whether or not the first dose is successfully chosen, later doses tend to concentrate about the ED_{50}. The advantages and disadvantages of the staircase method are discussed by Finney and some modifications are given.

2.3.2. Duration - A four-hour exposure period is recommended.

2.3.3. Test Conditions - The same recommendations as given for the initial screening should be used.

2.3.4. Plant Evaluation - Any foliar lesion produced by exposure to the test substance should be considered as a positive effect.

2.4 Results - When the tests are completed, the threshold concentration for a four-hour exposure should be estimated for the species most susceptible to the test substance. If the concentration that is predicted to occur in the ambient air (for a four-hour period) is less than the threshold concentration determined experimentally, the substance may be deemed acceptable and cleared for further production. If the threshold for injury is less than that expected in the ambient air, the substance may be deemed unacceptable and increased production would require more effective atmospheric controls. In evaluating the results of these tests, a number of compromises have been made which may prove later to be unsatisfactory. One of these is the conditions under which the tests are to be carried out. Relatively small differences in temperature or relative humidity can have drastic effects on the response of plants to a phytotoxicant. Both relative humidity and the frequency of precipitation will be significant factors in phytotoxic effects of particulate materials. The tests do not consider that plants respond differently to phytotoxicants at different ages or stages of development. The screening tests also assume that ambient exposures of only four hours will occur in the field or that the effects induced will be based upon total dose (time x concentration). In all probability, this is not true; thus, a concentration of 100 $\mu g/m^3$ for 4 hours (400 $\mu g/m^3 \cdot hr^{-1}$) will probably be of a different degree of phytotoxicity than 4 $\mu g/m^3$ for 100 hours (400 $\mu g/m^3 \cdot hr^{-1}$).

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3.15 PROBLEMS IN THE DEVELOPMENT OF SCREENING PROTOCOLS FOR AIR-BORNE POLLUTANTS TEST CHAMBERS

Two types of chambers have been used for studies on the effects of air pollution on plants: (1) the portable or fixed field chamber used to study the effects of pollution on field-grown plants, and (2) controlled-environment chamber for use in the laboratory or greenhouse, which allowed from minimal environmental control to highly sophisticated chambers with elaborate controls. Perhaps the first of these chambers was used by Schroeder and Schmitz-Dumont in Germany in 1896 (1). Haywood (2) described a portable chamber in 1908, and Wislicenus (1) used a sophisticated attached greenhouse. Many other chambers have been developed and used since the first ones 79 years ago (3,4,5,6,7,8,9,10,11,12, 13,14,15,16,17). Open-top field fumigation chambers have been introduced recently (16,17).

The first problem is that most of these chambers have not been built under sufficiently rigid specifications to be acceptable for studies on the effects of air pollution on plants (18,19). The commonly used chambers are deficient with respect to many parameters, including good environmental control, uniform air distribution, a one-pass circulation system which can provide filtered air to the chamber, and types of materials used in construction. Commercially-available plant growth chambers have not been designed for use in air pollution studies. Although they generally provide acceptable control of temperature, relative humidity, and photoperiod, they do not usually provide control over light intensity. This problem is common to studies with water- or soil-borne as well as air-borne pollutants, so it does not present a serious obstacle. Because most chambers have not been designed for air pollution studies, the means of introduction and uniform dispersion of gaseous or particulate substances are less than adequate. Not only must the concentration of a pollutant be uniform throughout the chamber, but the air flow must be uniform because pollutant uptake (and injury) of plants is related to both concentration and the amount of the pollutant which passes over the foliar surface (19,20,21). A second problem is that it is inescapable that some substances to be tested will have corrosive properties, and/or may absorb or react with the inner chamber surfaces. These potential problems emphasize the need for a chamber with inner surfaces as chemically inert as possible. Corrosion of chamber parts is obviously unsatisfactory, but even absorption onto surfaces can cause problems of monitoring and contamination.

Thirdly, because the most satisfactory air distribution system is a non-recirculating arrangement, proper venting of the chamber exhaust gases must be provided. Finally, the presence of ambient pollutants in the incoming air-stream can be unsatisfactory for two reasons: (1) ambient pollutants, such as ozone, are highly phytotoxic; and (2) the presence of ambient pollutants may potentiate or antagonize the phytotoxic action of a test chemical, resulting in invalid test results. This necessitates the introduction of an appropriate filter (such as activated charcoal) in the incoming air stream to remove ambient pollutants.

3.15.1 Introduction of Pollutant into the Chamber

These protocols will require the testing of two physical forms of air-borne chemicals: gas and particle. The introduction and maintenance of desired atmospheric concentrations of either type of pollutant present a number of problems which will be discussed in this and in the subsequent section.

A number of methods have been used to generate or introduce gaseous pollutants into chambers. The simplest method is represented by metering commercially-available bottled gas into the chamber through an appropriate valve. The gas is often diluted with an inert gas such as nitrogen when bottled. This method has been used successfully for sulfur dioxide, oxides of nitrogen, chlorine, ethylene, and other less common air pollutants. A second method involves the generation and bottling of the pollutant, followed by appropriate dilution and metering into the chamber. Peroxyacetyl nitrate is handled in this manner. A third method involves the generation of the pollutant as it is introduced into the chamber. This is best represented by the generation of ozone by passing oxygen or air across an electrical discharge. The generated ozone is then metered into the chamber at an appropriate rate. Another example of this approach might be the generation of nitrogen dioxide by reacting copper with nitric acid or of potassium bisulfite with a strong acid, but neither is used commonly because of the poor control over concentration. A fourth method involves the volatilization of an aqueous solution of a substance to produce a gas, and is used commonly for fumigating with HF. (22). Introduction of test substances as gases might be accomplished by adapting one of these methods or may require new technology.

Generation of aerosols (air-borne particles) is more difficult than gases. Information on methods of introduction and design of appropriate chambers is limited because there is no large body of literature (23). The Bacho Microparticle Classifier was recommended by Darley et al. (23) for the introduction of dry particles but it is no longer available and the Wright Dust Food Mechanism (L. Adams Ltd., London, England) is a satisfactory substitute. Chemicals soluble in water can be introduced as aerosols into chambers by the use of pneumatic nozzles (such as Sprayco Model #686C/RXH 11 AM assembly). The particle size and degree of hydration can be controlled by the rate of supply of the test liquid, by the air pressure to the nozzle, and by use of other appropriate nozzles. One serious problem with aerosol fumigations often is poor distribution to all parts of the chamber. This can be improved to a large extent by the use of a turntable to equalize the aerosol dosage and environmental conditions over the plants used in an exposure.

3.15.2 Monitoring of Pollutant

The methods of introduction and control of any fumigant are only as good as the method used to monitor the concentration in the chamber. Monitoring is composed of two important steps: Collection and analysis. Many methods of collection of gaseous pollutants have been used, depending upon the chemical and physical characteristics of the gas, available equipment, and desired accuracy and reproducibility of the methods. The gases have been collected in impingers and bubblers containing water, acid, alkali, or other solvent; or in dry collectors with treated filter paper, membrane filters, charcoal, or other media. In some cases, the gas has been passed directly through an appropriate cell where its concentration is measured by a direct physical means (e.g., infrared or ultraviolet spectroscopy).

Particles are also collected in impingers or bubblers containing water or other solvents. But they are most commonly collected with a high-volume sampler or filter holder on glass, membrane, or paper filters or with a paper sampler. The particle size distribution can be determined by use of cascade impactors such as the Andersen or a Lundgren Rotating Drum Impactor.

The use of proper equipment does not guarantee a proper result, however. Improper placement of the sampling probe (for gases) or device can give fallacious results, and for this reason, tests should be made to insure uniform mixing or dispersion of the pollutant. In the case of gases, the composition of the sampling probe can be very significant. Certain materials such as polyvinyl chloride should be avoided; other materials such as Teflon, glass, stainless steel, polyethylene, or polypropylene may be satisfactory for some materials but not for others. Some sampling problems can be solved by the use of a heated probe.

When lengthy exposures are required, the frequency of sampling is important. For example, a single 10-minute sample in a 24-hour fumigation would not be sufficient to determine whether the proper concentration had been attained or maintained. Obviously, the more frequent the sampling the better the control can be, assuming that a rapid analytical method is available.

It is difficult to perform exposures at a desired concentration if a rapid analytical method is not available that will allow adjustments in the rate of introduction of the pollutant periodically. The analytical method should also have a high order of accuracy and precision.

3.15.3 Dose-Response

What response should be measured? The response measured could be the production of any foliar lesion, and this would be the simplest approach. The response measured could also be an effect on the fresh or dry mass of the top of the plant. There are innumerable parameters that could be measured. But can one be certain that the response measured is a significant one with respect to the native or cultivated flora of the area? For example, the presence of a foliar lesion may have no measurable effect upon the growth or vitality of the plant, or on its intended use, unless, of course, the leaves of the plant are eaten or it is an ornamental. A reduction in the mass of the plant may be unimportant if it is a potato, carrot, or beet and the tuber or root yield has not been affected. On the other hand, exposure to a chemical during the testing period may produce no foliar lesions or no affect on the mass of the plant, and produce an important effect. For example, a chemical may alter the nutritional composition of the plant; induce sterility by affecting pollen viability or fertilization; or through direct absorption or metabolism, be toxic to foraging animals, insects, birds, etc.

What duration of exposure should be used? It is probably impractical to require long-term exposures with test chemicals for many reasons: (1) it may be economically unfeasible; (2) the longer the exposure, the more sophisticated the equipment required to maintain good control; (3) most controlled environment chambers will not support normal growth of many species for a long duration.

Should a dose-response curve be established for each chemical to be tested? The difficulties in the establishment of dose-response curves should be apparent from the preceding discussions, but there are other problems not yet discussed. One of these is that a given "dose" (a measured concentration of a toxicant for a known duration of time) of an airborne pollutant may produce different effects on the plant, depending upon how it is applied. For example, the phytotoxicity of, say, 1 ppm of a compound for 168 hours (7 days) may be completely different from 168 ppm for 1 hour, but the dose is the same (168 ppm hrs.). The lack of reciprocity of a given dose applied in different ways has been shown experimentally for SO_2 (24), ozone (25), and HF (26). The difference between the two types of exposures is the difference between acute and chronic injury. In the former case (high concentration-short duration), the toxicant may essentially "swamp" the metabolic systems of the plant which have no opportunity to accommodate to this insult and major injury may occur. In the latter case (low concentration-long duration), the plant may detoxify the pollutant by metabolic change (e.g., sulfur dioxide to sulfate), insolubilization (e.g., HF to CaF), changes in the metabolic pathways of the plant cell, or by other means (27), (28) and injury may be slight and difficult to measure.

Because fumigations in the field are more likely to result in low concentrations of pollutant for long durations than the more extreme insult, one must question the value of establishing screening procedures based upon a type of exposure that is least likely to occur. On the other hand, long-term, low-level, fumigations are prohibitive economically and pose technical problems that have not yet been satisfactorily resolved.

3.15.4 Factors Which Affect Response of the Plant to a Pollutant

The response of the plant to air-borne, as well as water- and soil-borne pollutants, is affected by many factors. Although most of these are important for the three types of pollutant, some are more important to the air-borne pollutants.

First, there are a number of climatic factors which influence plant response. Generally, the phytotoxicity of the pollutant increases with temperature (29), (30), and light intensity (33). The photoperiod in relation to the time of exposure of the plant to ozone or peroxyacetyl nitrate is also important (33). In the case of air-borne pollutants, precipitation or the presence of free water on foliar surfaces can be a determining factor in whether or not injury will occur from a given exposure. This is especially important in the case of particulate materials which reside on the plant surfaces, but can also be important with gaseous pollutants. In the case of particulate materials, the occurrence of light precipitation or dew can solubilize the particles and aid in foliar penetration, thus increasing the potential for injury. Heavy precipitation can remove the particles from the plant surfaces and, in some cases,ses, remove phytotoxic materials that were adsorbed to the surface or absorbed as a gas and excreted (34).

Second, are several edaphic factors which affect the response of the plant to pollutants introduced through the air, water, or soil. These include the nutrient status of the plant, a subject about which there is only limited information, and soil moisture. The presence of adequate soil moisture generally favors the injury of plants by air-borne pollutants (35).

Third, factors associated with the pollutant itself are important in determining the production of plant injury. These factors include concentration, duration of exposure, recurrency of exposure, physical and chemical properties of the pollutant, and the presence of other pollutants during the exposure period. The importance of concentration and duration of exposure have been discussed earlier. The recurrency (or frequency) of exposure is also a significant factor with respect to the response of the plant. Obviously, the more recurrent the exposure, the greater the probability of producing injury to the receptor. But recurrent exposures can be less phytotoxic than continuous ones because the plant has time to accommodate to this intrusion by detoxification, excretion, or metabolism. The types and recurrency of exposures that will occur near any manufacturing facility will be determined by the nature of the manufacturing processes, and by meteorological and topographical characteristics of the area. The importance of the physical and chemical nature of the pollutant has been discussed briefly in an earlier section, but several important features should be mentioned. With respect to particulate substances, phytotoxicity is closely associated with particle size and degree of hydration of the particle. Very small particles (ca. $<1\ \mu$) may not deposit on vegetation but may act as a gas. Larger particles will impact on the plant but will not be transported over distances as great as smaller particles. Small particles of relatively insoluble materials will be more soluble than large particles. Hydrated particles are more immediately phytotoxic than dry particles, but the presence of surface moisture as dew or after a rainfall would eliminate this difference. Finally, the presence of other pollutants before, during, or after exposure to an air pollutant may alter its phytotoxicity, and the occurrence of synergistic and antagonistic effects are known (36,37,38,39,40,41,42). There is a high probability that other pollutants will also occur in the atmosphere near any chemical manufacturing facility, either from other chemical processes, nearby industries, long-range transport, etc., and the possibility of interactive effects should be considered in the evaluation of potential effects on plants.

Fourth, the response of a plant to a toxicant will be influenced by several *biological* factors, including the *stage of plant development*, the *age of the leaf*, and *heredity*. During the ontogeny of a plant, its susceptibility to environmental stresses, including air pollution, will change. The direction and extent of this change will depend upon the plant and the pollutant.

Young plants are usually more susceptible to air-borne pollutants than older plants, and the flowering stage of plants can be a particularly susceptible period. As the leaf ages, its susceptibility to pollutants changes, decreasing for some, increasing for others. There is a wide disparity between plants in their susceptibility to any pollutant, but the order of susceptibility among species is different for each pollutant. Thus, a test plant may be a susceptible receptor for one chemical and a resistant receptor for another. Even within a field population of plants, such as pines, wide differences will be found in the susceptibility of the various genotypes to any one pollutant (35).

3.15.5 Indirect Effects on the Pollutant-Plant Interaction

Although the possible indirect effects of air-borne pollutants are not a necessary component in the preparation of the screening methods, the contractor and user of these methods should recognize the fact that these effects occur, and may produce effects that are more important ecologically than the direct effect of the pollutant alone. The uptake and accumulation of some pollutants may have little or no direct effect on the plant, but may have serious consequences on other components of the biotic environment. For example, although accumulation of fluoride, lead, mercury, cadmium, other heavy metals, and nuclides can affect many plants, depending upon species, stage of development, environmental factors, and other circumstances, ingestion of the plant by foraging animals (cattle and other herbivores), birds, or insects can cause disease, such as fluorosis in the case of fluoride (43). The loss of leaves through natural processes can transfer accumulated toxicants to the soil, where an effect may be produced in the soil microbial population. Changes

Changes that occur in metabolism and certain volatile constituents may affect the suitability of the plant as a habitat for destructive insects or plant diseases. Although little is known of this area, the plant may metabolize a substance to a form more toxic to the plant or the biotoc environment. The metabolism of inorganic fluoride ion to monofluoroacetic acid in a number of African, Australian, and Brazilian species has been responsible for the innumerable cattle fatalities (44).

Finally, the pollutant may affect the intended use of the plant, whether for food, fiber, or for aesthetic purposes. Thus, a subtle effect, such as small difference in plant form may make the plant unsuitable for mechanical harvesting and decrease its value (45).

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4. SUMMARY

The herbicide literature has provided information on the mode of action of chemicals from a variety of structural classes on numerous types of plants. The majority of available information on the effects of chemicals on plants has come from research on herbicides and other pesticides. Although research on air pollution effects on plants has provided additional insight into the problem, these studies have been restricted to a relatively small group of pollutants and their effects on specific receptor species.

The important crop plants provide most of the data base of test species. Studies on naturally growing plants in the ecosystem have been limited to forest management techniques and the like and have not focused on toxicity per se. Although methods for determining chemical effects on trees are reported, the time involved in testing from seed to seed precludes their use as test plants.

Because it would be impossible as well as impractical to test every significant species and strain against even a single chemical, a limited group of preliminary test species has been recommended. These were selected on the basis of their importance as crops, their susceptibility to chemicals, and certain anatomical and physiological characteristics. Bioassay methods have been selected as the procedures, since they more closely reflect natural conditions than do techniques involving biochemical or cytological examination. It is recommended that the extent of plant toxicity testing be directly proportional to the quantity of a chemical which is to be manufactured and the resulting hazard to the environments.

5. CONCLUSIONS

5.1 STATE OF THE ART

5.1.1 Herbicide research, air pollution studies on plants, and investigations of the aquatic algae and their environments provided substantial quantities of data for this report. The results of herbicide research have provided significant information pertaining to chemical effects on crop plants. These studies focused on the differential toxicity between wanted and unwanted species. The bulk of industrial, academic, and government research has been oriented toward crop protection _by_ chemicals rather _from_ chemicals.

5.1.2 Air pollution studies have provided data on the effects of some pollutants on specific species. These receptor plants were selected because of their known responses and are often non-representative of either naturally-growing or commercial crop plants as in the case of Bel-W_3 tobacco. Ecological studies of chemical effects on plants have been limited mainly to aquatic environments and have been performed principally with algae both individually and in communities. The understanding of the ecological significance of interrelationships among aquatic algae and diatoms in the food chain is greater than that achieved in the case of aquatic vascular plants.

5.1.3 Terrestrial plant communities present several complex problems. There are hundreds of major communities throughout the United States and the problem is complicated by the number of species involved. Therefore, emphasis should be placed on species of ecological importance. When studying a particular ecosystem, species whould be selected from the study site.

5.2 Test Methods Selected

5.2.1 Bioassay type determination of chemical effects on plants should be performed by bioassay techniques during the critical stages of the plant's life cycle. These tests were chosen because they assess the effects of toxic chemicals rather than study the mechanism of action.

5.2.2 Preliminary sequence testing should be performed in a laboratory incubator/growth chamber prior to greenhouse and field testing. For incubator, growth chamber, and greenhouse testing, terrestrial plants should be grown in sand or vermiculite. Temperautre, humidity, light intensity, nutrients, and duration of exposure should be standardized. Application of chemicals may be pre-plant, pre-emergence, post-plant, or post-emergence.

5.2.3 Aquatic plant testing has been limited to algae. Under controlled conditions both static and flow through techniques are recommended.

5.2.4 When the hazardous substance is airborne, air pollution type testing should be used. These tests should be conducted under controlled conditions in fumigation chambers. Development of a routine bioassay for effects of typical air pollutants and other airborne chemicals on plants does not seem feasible at present due to the inadequacy of existing methodology.

5.3 Species Selection

Criteria for species selection included: knowledge of the plant's physiology, commercial and/or ecological significance, sensitivity, resistivity, and availability of uniform strains. No single strain or species can be considered to be the most susceptible or the most resistant to chemical insult in general.

6. RECOMMENDATIONS

Pilot studies should be performed to test the validity of the procedures recommended in this report. To determine their reproducibility, these should be conducted simultaneously in several laboratories. Chemical standards should include, but not be limited to, a representative of each major category of herbicides and plant growth regulators listed in Table 2.2,p. 2-12, Mode of Action by Various Chemicals Disrupt Plant Growth.

1. Lipid Synthesis Inhibition; structural organization disrupted.

    EPTC

2 & 3. Cell Membrane Disruption/Electron Transport Inhibition

    diquat

    paraquat

4. Enzyme System Inhibition

    DSMA

5. Oxidative Phosphorylation Uncoupling

    PCP

6. Photosynthetic Electron Transport Inhibition

    diuron

    atrazine

7. Carotenoid Synthesis Inhibition

    amitrole

8. Cellular or Nuclear Division Inhibition

chlorpropham

9. Indoleacetic Acid Mimicking

2,4-D

picloram

10. Indoleacetic Acid Transport Interference

naptalam

11. Gibberlin Inhibition

phosphon

12. Affect Ethylene Production

ethephon

13. Combination With Proteins

dalapon

14. Nitrogen Metabolism Disrupter

sodium chlorate

15. Mode of Action Unknown

dichlobenil

diphenamid

bensulide

Should any of these effects fail to be reflected by the species suggested, additional species may be studied. Following successful pilot testing, a list of industrial (non-agricultural) chemicals should be subjected to testing based on the quantity manufactured, the environmental release, and predicted hazards to the environment.

Throughout this project, two major deficiencies in current knowledge have become apparent. These are the inadequate utilization of information and research results tangentially related to pollutant effects on plant growth, i. e., herbicidal research, and the lack of information pertaining to chemical effects on natural plant communities. Therefore, new research efforts are encouraged with respect to these factors. The following are recommendations:

- The development of research procedures to evaluate the effects of chemical toxicants on natural plant communities and ecosystems. These would cover single growing seasons as well as long term studies that encompass the complete life cycles of perennial herbaceous and woody plants. Exposure routes should include soil, water, aerosols, and vapor.

- The development of correlative procedures for elucidating relationships of growth chamber, greenhouse, and field plot tests to natural ecosystems. This would provide a basis for extrapolation of effects found in small scale systems to natural plant communities.

- The development of compressed life cycle procedures so that potential effects may evolve in short time spans. This would be applicable to forests and perennial vegetation communities.

APPENDIX A

APPENDIX A

INTERVIEWS

In the course of gathering information for this report, leading members of the scientific community were interviewed for first-hand information. Not all of those contacted were available for personal consultation; several were interviewed solely by telephone. A number of those interviewed personally requested that they not be quoted or identified. In deference to their wishes, a listing of only the organizations visited is presented. The figure in parentheses indicates the number of individuals interviewed.

Ag-Organics Dept. (4)
Dow Chemical Co.
Walnut Creek, CA 94598

Agricultural Chemical Div. (1)
Amchem Products Inc.
Ambler, PA 19002

Argicultural Div. (2)
CIBA-GEIGY Corp.
Greensboro, NC 27409

Agricultural Division (2)
Shell Chemical Co.
Modesto, CA 94598

Agricultural Research Service (7)
U.S. Dept. of Agriculture
Beltsville, MD 20705

Biological Research Center (1)
ICI-America Inc.
Goldsboro, NC 27530

Dept. of Crop Science, Botany (1)
& Forestry
North Carolina State University
Raleigh, NC 27607

Dept of Entomology (1)
Pesticide Research Laboratory
Pennsylvania State University
University Park, PA 16802

Dept. of Forestry (1)
Oregon State University
Corvallis, OR 97331

Dept. of Limnology (1)
Academy of Natural Sciences
19 & Benjamin Franklin Parkway
Philadelphia, PA 19103

Dept. of Plant Biology (2)
Cook College
Rutgers University
New Brunswick, NJ 08903

Dept. of Plant Science (1)
University of California
Riverside, CA 92507

EPA National Environmental (4)
Res. Lab.
Air Pollution Res. Lab
Corvallis, OR 97331

EPA Technical Services (1)
Division
Office of Pesticide Programs
Corvallis, OR 97331

International Plant Protection (2)
Center and Dept. of Agronomic Crop
Science
Oregon State Univ.
Corvallis, OR 97331

Pacific Northwest Research (3)
Lab.
Environmental Protection
Agency
Corvallis, OR 97331

Southern Region ARS-USDA (2)
Plant Pathology Dept.
North Carolina State Univ.
Raleigh, NC 27607

Southeastern Regional (1)
Research Lab.
Environmental Protection
Agency
Athens, GA 30601

Statewide Air Pollution (4)
Center
University of California
Riverside, CA 92502

APPENDIX B

TAXONOMIC LISTING OF SUGGESTED TEST SPECIES

SOIL BACTERIA

Schizomycophyta; Schizomycetes; Pseudomondales; Pseudomonadinaea; Nitrobacteraceae; *Nitrosomonas* and *Nitrobacter*.

BLUE-GREEN ALAGE

Myxophyta (Cyanophyta); Cyanophyceae; Nostocales; Nostocaceae; *Anabaena flor-aquae*.

Myxophyta (Cyanophyta); Cyanophyceae; Chroocooccales; Chroococcaceae; *Anacystis Cyanea*.

SOIL FUNGI

Eumycophyta (Myxomycophyta); Deuteromycetes; Moniliales; Moniliaceae *Tricoderma viride*.

GREEN ALAGE

Chlorophyta; Chlorococcales; Oocystaceae; *Selenastrum capricornutum*.

AQUATIC VASCULAR PLANTS

Magnoliophyta; Magnoliatae; Ronales; Nymphaeceae; *Cabomba caroliniana*.

Duckweed

Magnoliophyta; Liliatae; Arales; Lemnaceae; *Lemna minor*.

Magnoliophyta; Liliatae; Arales; Lemnaceae; *Lemna gibba*.

Waterweed

Magnoliophyta; Liliatae; Hydrocharitales; Hydrocharitaceae; *Elodea canadensis*.

TERRESTRIAL VASCULAR PLANTS

CORN

Magnoliophyta (Angiospermae); Liliatae (Monocotyledonae); Cyperales; Gramineae; Panicoideae; Tripsaceae; *Zea mays;* 'Butter' and 'Sugar'.

OATS

Magnoliophyta (Angiospermae); Liliatae (Monocotyledonae); Cyperales; Gramineae; Poacoideae; *Avena sativa;* 'Clintford'.

RYEGRASS

Magnoliophyta (Angiospermae); Liliatae (Monocotyledoneae); Cyperales; Gramineae; Poacoideae; Hordeae; *Lolium perenne;* 'Manhattan'.

BEAN

Magnoliophyta (Angiospermae); Magnoliatae (Dicotyledonae); Rosales; Legumiosae; Fabacea; *Phaseolus vulgaris* 'Pinto'.

CUCUMBER

Magnoliophyta (Angiospermae); Magnoliatae (Dicotyledonae); Violales; Cucurbitaceae; *Cucumis sativus;* 'Marketer'.

TOMATO

Magnoliophyta (Angiospermae); Magnoliatae (Dicotyledonae); Polemoniales; Solanaceae; *Lycopersicon esculentum;* 'Rutgers'.

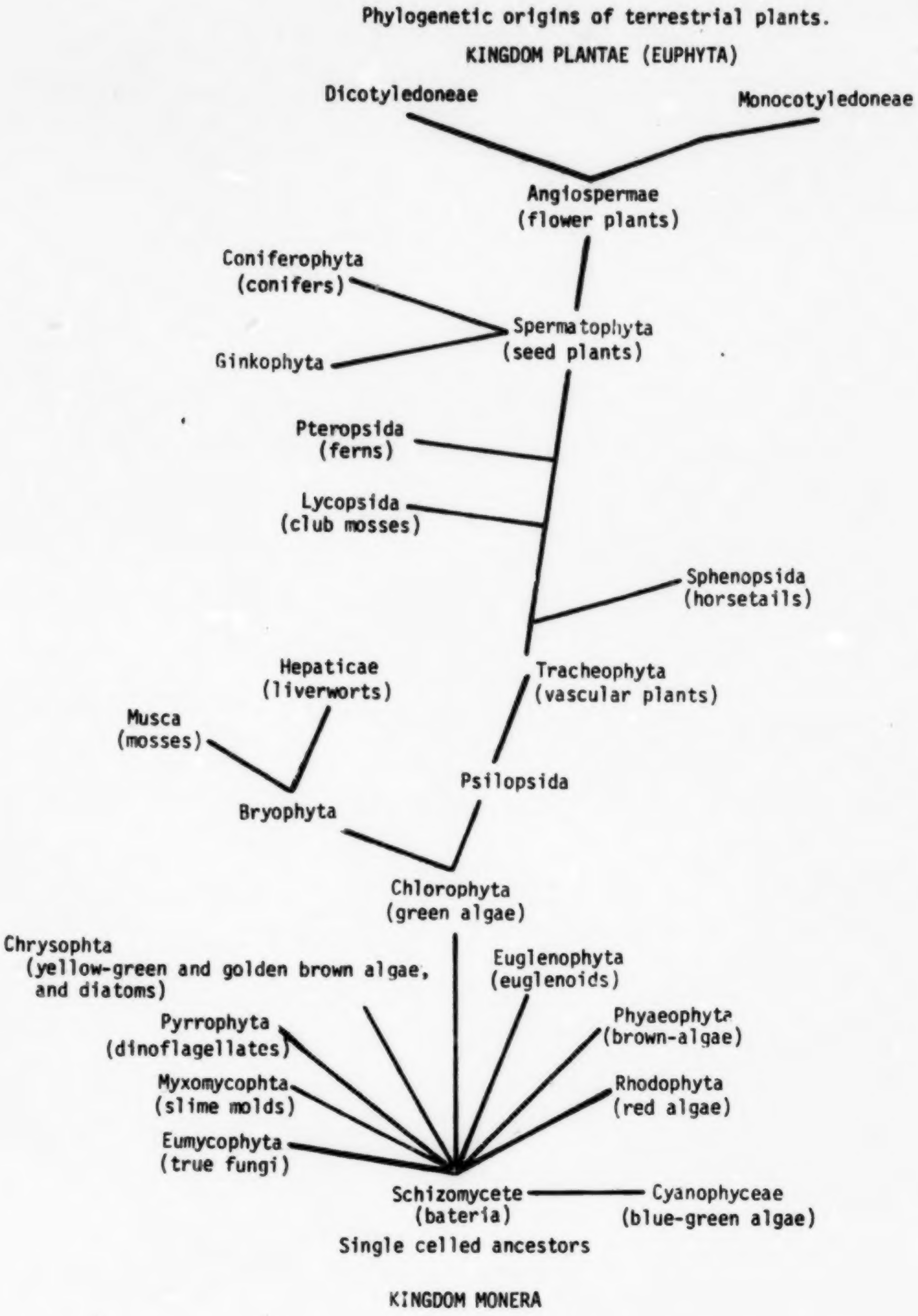
Phylogenetic origins of terrestrial plants.
KINGDOM PLANTAE (EUPHYTA)
Dicotyledoneae
Monocotyledoneae
Angiospermae
(flower plants)
Coniferophyta
(conifers)
Spermatophyta
(seed plants)
Ginkophyta
Pteropsida
(ferns)
Lycopsida
(club mosses)
Sphenopsida
(horsetails)
Hepaticae
(liverworts)
Tracheophyta
(vascular plants)
Musca
(mosses)
Psilopsida
Bryophyta
Chlorophyta
(green algae)
Chrysophta
(yellow-green and golden brown algae,
and diatoms)
Euglenophyta
(euglenoids)
Pyrrophyta
(dinoflagellates)
Phyaeophyta
(brown-algae)
Myxomycophta
(slime molds)
Rhodophyta
(red algae)
Eumycophyta
(true fungi)
Schizomycete
(bateria)
Cyanophyceae
(blue-green algae)
Single celled ancestors
KINGDOM MONERA

Figure 2. Phylogenetic Origins of Terrestrial Plants

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